INHIBITION OF PLATELET AND VASCULAR SMOOTH MUSCLE FUNCTION BY CYCLIC NUCLEOTIDES: SYNERGISM BETWEEN ACTIVATORS OF ADENYLYL AND GUANYLYL CYCLASES

By

Donald Hector Maurice, B.Sc.

A thesis
Submitted to the School of Graduate Studies
in Partial Fulfilment of the Requirements
for the Degree
Doctor of Philosophy

McMaster University

(c) Copyright by Donald Hector Maurice, February 1991

SYNERGISM BETWEEN ACTIVATORS OF ADENYLYL AND GUANYLYL CYCLASES

DOCTOR OF PHILOSOPHY (1991)
(Medical Sciences)

McMASTER UNIVERSITY
Hamilton, Ontario

TITLE: Inhibition of platelet and vascular smooth muscle function by cyclic nucleotides: synergism between activators of adenylyl and guanylyl cyclases

AUTHOR: Donald Hector Maurice, B.Sc. (Carleton University)

SUPERVISOR; Richard J. Haslam, Ph.D., M.A.

NUMBER OF PAGES: xxiv, 352

ABSTRACT

In this thesis, I have investigated the inhibitory effects of activators of guanylyl cyclase and adenylyl cyclase on rabbit platelet and rat vascular smooth muscle (VSM) function, with particular reference to the synergistic interactions between these two classes of compounds.

In platelets, the activators of guanylyl cyclase used were sodium nitroprusside (SNP) and SIN-1 (the active metabolite of molsidomine). PGE_1, a functional analogue of prostacyclin (PGI_2), and adenosine were the activators of platelet adenylyl cyclase studied. Changes in cyclic [^{3}H]nucleotides were measured in platelets prelabelled with [^{3}H]guanine and [^{3}H]adenine. Incubation of labelled platelets with SNP or SIN-1 caused inhibitions of platelet function which were associated with large dose-dependent increases in [^{3}H]cGMP and 1.4 to 3.0-fold increases in [^{3}H]cAMP. However, addition of either SNP or SIN-1 with PGE_1 or adenosine, at concentrations of the latter that had little effect alone, caused much larger increases in [^{3}H]cAMP and a greatly enhanced the inhibition of platelet function. Experiments with an adenylyl cyclase inhibitor, 2',5'-dideoxyadenosine (DDA), suggested that these synergistic interactions depend on an enhanced accumulation of cAMP. Hemoglobin inhibited all nitrovasodilator-mediated effects. Cilostamide, a selective inhibitor of platelet cGMP-inhibited cAMP phosphodiesterase

(cGI-PDE), had effects identical to those of SNP, suggesting that the actions of the latter depend on inhibition of the same enzyme. Also, the results obtained with M&B 22,948, a cGMP-selective PDE inhibitor, strongly suggest that the increases in platelet [^{3}H]cAMP caused by nitrovasodilators, in the presence or absence of activators of adenylyl cyclase, are mediated by the inhibition of cAMP breakdown by cGMP.

To determine if a similar synergism is seen in vascular smooth muscle (VSM), the interactions between isoproterenol and either nitrovasodilators or cAMP-PDE inhibitors were studied. Although both SNP and SIN-1 and cAMP-PDE inhibitors caused significant reductions in the IC_{50} value for relaxation of precontracted rat aortic smooth muscle by isoproterenol, more marked effects were observed when these compounds were added prior to induction of contraction (by phenylephrine). Thus, synergism between nitrovasodilators and isoproterenol could be detected in VSM. This action of the nitrovasodilators resembled that of inhibitors of cAMP PDE. Consistent with this, concentrations of SNP that caused synergistic inhibitions of contraction with isoproterenol enhanced the increases in cAMP caused by the latter. Although a low concentration of SNP increased cGMP but not cAMP, a higher concentration caused a significant increase in the levels of both cyclic nucleotides. However, the increases in cGMP caused by SNP were unaffected by isoproterenol. From these studies, I conclude that the synergistic inhibitory effects of activators of guanylyl and adenylyl cyclases observed in platelets and VSMC are likely to be

mediated by cAMP, and that the synergistic accumulation of cAMP results from an inhibition of cAMP breakdown by cGMP, probably acting at the cGI-PDE.

A fast and sensitive prelabelling method was established for the measurement of cyclic nucleotides in cultured vascular smooth muscle cells (VSMC) from rat aorta. Using this method, agonist-induced changes in the cyclic nucleotide levels of two different aortic VSMC lines were studied. However, the effects of activators of adenylyl and guanylyl cyclases observed in these cell lines differed from those seen in intact aorta, probably as a result of the loss of key regulatory enzymes during culture.

ACKNOWLEDGMENTS

I wish to thank Dr. R.J. Haslam for his guidance and constructive criticism during my period of study in his laboratory. I have found Dr. Haslam to be an excellent scientist and a trustworthy supervisor. I would also like to thank Dr. R.M.K.W. Lee for the cultured vascular smooth muscle cells and the members of my supervisory committee (Drs. Denis Crankshaw and John Vickers) for their guidance. I am also grateful for the financial assistance granted me by the graduate student scholarship programme (OGS) of the Province of Ontario and the Ontario Heart Foundation. I also wish to thank my friends (Monica, Janet, Martine, Mary, Raj, Claudio, Arlene, Jim, Kim, Wanda, Bruno, Renald, Derryl, Steve, Robert, Jean-Pierre, Frank, and Luke) without whose friendship the completion of this work would have been much less enjoyable. Also, I want to thank my friend David Kitson, who I wish had decided to stay. I also want to thank my wife Samina for her love and understanding during these last four years. Je me dois aussi de remercier mes parents, Daniel et Irene Maurice, pour leurs encouragement durant cette période d'étude.

TABLE OF CONTENTS

Chapter 1 Introduction 1

1.1. Platelet structure 2

1.2. Platelet physiology and pharmacology 3

1.2.1. Thrombin 6

1.2.2. Platelet activating factor 7

1.3. Signal transduction pathways in platelet activation 8

1.3.1. General considerations 8

1.3.2. GTP-binding proteins 12

1.3.3. Phospholipase C 13

1.3.4. Phospholipase A_2 15

1.4.5. Protein kinase C 16

1.4. Role of Ca^{2+} in platelet aggregation 17

1.4.1. Ca^{2+} influx 18

1.4.2. Ca^{2+} release from the dense tubular system 20

1.5. Inhibition of platelet function by cyclic nucleotides 20

1.5.1. cAMP and cGMP mediated phosphorylation of platelet proteins 21

1.5.2. Effects of cAMP and cGMP on platelet $[Ca^{2+}{}_i]$ 24

1.5.2.1. Effects on agonist binding 25

1.5.2.2. Effects on platelet plasma membrane Ca^{2+} channels 25

1.5.2.3. Effects on phosphatidylinositol hydrolysis 26

1.5.2.4. Effects of cyclic nucleotides on Ca^{2+} kinetics 27

1.5.2.5. cAMP and cGMP and the function of contractile proteins 28

1.6. Vascular smooth muscle structure 29

1.7. Vascular smooth muscle physiology and pharmacology 30

1.8. Contraction of vascular smooth muscle 32

1.8.1. Role of Ca^{2+} in smooth muscle contraction 32

1.8.2. Ca^{2+} influx 33

1.8.3. Ca^{2+} release 35

1.8.4. Role of MLC and MLCK in vascular smooth muscle contraction 37

1.8.5. PKC-mediated phosphorylation and sustained VSM contraction 39

1.9. Inhibition of VSM function by cyclic nucleotides 40

1.9.1. cAMP- and cGMP-mediated protein phosphorylation in VSMC 40

1.9.2. Effects of cAMP and cGMP on $[Ca^{2+}{}_i]$ in VSMC 42

1.9.2.1. Effects of cAMP and cGMP on Ca^{2+} entry into VSMC 42

1.9.2.2. Effects of cAMP and cGMP on phosphatidylinositol hydrolysis and the mobilization of Ca^{2+} by $Ins(1,4,5)P_3$ 43

1.9.2.3. Effects of cAMP and cGMP on Ca^{2+} removal from the cytosol 44

1.9.2.4. Effects on the contractile apparatus 46

1.10. Adenylyl cyclase system 47

1.10.1. Receptors 47

1.10.2. Receptor-G-protein interactions 50

1.10.3. Interaction of G_s and G_i with and the catalytic subunit of adenylyl cyclase 51

1.10.4. Catalyst 52

1.11. cAMP-dependent protein kinase (cAMP-PK) 52

1.12. Synthesis and function of cGMP 55

1.12.1. Synthesis of cGMP 55

1.12.2. Soluble guanylyl cyclase 55

1.12.3. Particulate guanylyl cyclase 59

1.13. cGMP-dependent protein kinase (cGMP-PK) 61

1.13.1. cGMP binding sites 63

1.13.2. Catalytic domain 64

1.13.3. Activation 65

1.14. Cyclic nucleotide phosphodiesterases 66

1.14.1. Type I: Ca^{2+}/calmodulin-activated phosphodiesterases (Ca^{2+}/calmodulin-PDE) 68

1.14.2. Type II: cGMP-stimulated cAMP phosphodiesterases (cGS-PDE) 70

1.14.3. Type III: cGMP-inhibited cAMP phosphodisterases (cGI-PDE) 72

1.14.3.1. Platelet cGI-PDE 73

1.14.3.2. VSM cGI-PDE 75

1.14.3.3. Cardiac cGI-PDE 75

1.14.3.4. Adipocyte cGI-PDE 76

1.14.3.5. "Dense-vesicle" PDE 77

1.14.4. Type IV: Ro 20-1724 inhibited cAMP-phosphodiesterases; (RoI-PDE) 78

1.14.5. Type V: cGMP-specific phosphodiesterases (cGMP-specific PDE) 79

1.14.5.1. Lung and platelet cGMP-specific PDE 79

1.14.5.2. The smooth muscle enzyme 80

1.14.5.3. Rod and cone cGMP-specific PDEs 81

1.14.6. Structure/function relationships between PDEs of different families 82

1.15. Thesis objectives 82

Chapter 2. Materials and Methods 83

2.1. Materials 84

2.1.1. Animals 84

2.1.2. Radiochemicals and scintillants 84

2.1.3. Fine chemicals and pharmacological agents 84

2.1.4. Chromatographic supplies 85

2.1.5. Tissue culture materials and other supplies 85

2.1.6. Pharmacological agents supplied as gifts 86

2.2. Methods 86

2.2.1. Preparation of washed rabbit platelets 86

2.2.2. Preparation of washed rat platelets 87

2.2.3. Labelling of washed platelets 87

2.2.4. Platelet incubation conditions 89

2.2.5. *Measurement of platelet aggregation and degranulation* 89

2.2.6. *Subculture and labelling of VSMC* 90

2.2.7. *VSMC incubation conditions* 91

2.2.8. *Determination of [^{3}H]cGMP and [^{3}H]cAMP in acidified cell extracts* 91

2.2.9. *Determination of the radiochemical purity of cyclic [^{3}H]nucleotides isolated from labelled platelets and VSMC by the dual column procedure* 92

2.2.10. *Measurement of [^{3}H]GTP and [^{3}H]ATP in labelled platelets* 94

2.2.11. *Isolation of [^{3}H]ATP and [^{3}H]GTP from cultured VSMC* 96

2.2.12. *Purification, separation and concentration of platelet and cultured VSMC cyclic nucleotides for radioimmunoassay* 97

2.2.13. *Determination of platelet and vascular smooth muscle cAMP and cGMP by radioimmunoassay* 98

2.2.14. *Preparation of rat aortic rings* 101

2.2.15. *Determination of the optimal pre-load tension* 102

2.2.16. *Studies on the relaxation of aortic smooth muscle by isoproterenol* 103

2.2.17. *Studies on inhibition of the contraction of aortic smooth muscle* 104

2.2.18. *Analysis of concentration-response curves* 105

2.2.19. *Cyclic nucleotide measurements in rat aortic smooth muscle* 106

2.2.20. Protein assays 107

2.2.21. Statistics 109

Chapter 3. Inhibition of platelet function by nitrovasodilators and activators of adenylyl cyclase 123

3.3. Introduction 124

3.1.1. Effects of vasodilators on platelet and vascular smooth muscle function 124

3.1.2. Objectives 127

3.2. Results 127

3.2.1. Prelabelling technique for simultaneous measurement of [^{3}H]cAMP and [^{3}H]cGMP; purity of cyclic [^{3}H]nucleotides and comparison with radioimmunoassays 128

3.2.2. Effects of different concentrations of SNP on rabbit platelets 131

3.2.3. Influence of DDA on the effects of SNP on rabbit platelets 132

3.2.4. Synergistic effects of SNP and PGE_1 on rabbit platelets function 133

3.2.5. Effects of SNP and PGE_1 on rabbit platelet [^{3}H]cyclic nucleotides 134

3.2.6. Effects of DDA on the synergistic effects of PGE_1 135

and SNP

3.2.7. Effects of SIN-1 on rabbit platelet aggregation 135

3.2.8. Effects of SIN-1 and PGE_1 on cyclic [^{3}H]nucleotides in rabbit platelets 136

3.2.9. Effects of DDA on the synergistic action of SIN-1 and PGE_1 on rabbit platelets 137

3.2.10. Synergistic effects of nitrovasodilators and adenosine on rabbit platelets 138

3.2.11. Effects of hemoglobin on the actions of the nitrovasodilators on rabbit platelets 140

3.2.12. Comparison of the effects of cilostamide and SNP in rabbit platelets 142

3.2.13. Effects of M&B 22,948 on cyclic [^{3}H]nucleotides in rabbit platelets 143

3.2.14. Effects of SNP and PGE_1 on rat platelet aggregation 145

3.2.15. Effects of SNP and PGE_1 on rat platelet cyclic [^{3}H]nucleotides 146

3.2.16. Comparison of the effects of cilostamide and SNP in rat platelets 147

3.3. Discussion 148

3.3.1. Cyclic nucleotide measurements in platelets 149

3.3.2. Pharmacological impact on platelet function of the nitrovasodilator-induced increases in platelet [^{3}H]cAMP 150

3.3.3. Molecular basis for the nitrovasodilator-induced 151

increases in platelet [^{3}H]cAMP
3.3.4. Physiological significance of the interaction between activators of adenylyl and guanylyl cyclases 155
3.4. Summary 158

Chapter 4. Inhibition of vascular smooth muscle function by nitrovasodilators and isoproterenol 196
4.1. Introduction 197
4.1.1. Effects of nitrovasodilators on platelet and VSM 197
4.1.2. Interactions between activators of adenylyl and and guanylyl cyclases 197
4.1.3. Elements necessary for synergism between activators of adenylyl and guanylyl cyclases are present in vascular smooth muscle 198
4.1.4. Objectives 199
4.2. Results 200
4.2.1. Relaxation of precontracted rat aortic smooth muscle by vasodilators 200
4.2.2. Effects of vasodilators on the contraction of rat aortic smooth muscle 204
4.2.3. Effects of SNP and isoproterenol on cyclic nucleotide levels in rat aortic smooth muscle 206
4.3. Discussion 208
4.3.1. Synergistic interactions between isoproterenol and nitrovasodilators or cAMP PDE inhibitors 208

4.3.2. Potentiation by SNP of isoproterenol-induced increases in rat aortic smooth muscle cAMP is consistent with effects of these agents on function 213

4.4. Summary 217

Chapter 5. Agonist-induced changes in cyclic nucleotides in cultured VSMC 247

5.1. Introduction 248

5.1.1. Objectives 249

5.2. Results 249

5.2.1. Measurement of cyclic nucleotides in cultured VSMC 249

5.2.2. Labelling of cultured VSMC by [^{3}H]hypoxanthine 251

5.2.3. Radioisotopic purity of cyclic nucleotides isolated from prelabelled cultured VSMC 253

5.2.4. Validation of the prelabelling assay using radioimmunoassays 254

5.2.5. Effects of isoproterenol on cyclic nucleotide levels in cultured VSMC (W1) 255

5.2.6. Effects of SNP on the cyclic nucleotide levels in cultured VSMC (W1) 256

5.2.7. Effects of SNP, cilostamide and Ro 20-1724 on isoproterenol-induced increases in [^{3}H]cAMP in cultured VSMC (W1) 257

5.2.8. Effects of adenosine on the cyclic nucleotide levels 258

cultured VSMC (W2)

5.2.9. Effects of SNP on cyclic nucleotide levels in cultured VSMC (W2) 259

5.2.10. Effects of SNP, cilostamide and Ro 20-1724 on adenosine-induced increases in [^{3}H]cAMP in cultured VSMC (W2) 259

5.3. Discussion 260

5.4. Summary 267

Chapter 6. General Discussion 293

6.1 General discussion 294

6.1.1. Novel findings reported in this thesis 294

6.1.2. Role(s) of cyclic nucleotides in mediating inhibitory effects 296

6.1.3. Physiological significance of synergism between nitrovasodilators and activators of adenylyl cyclase in platelet and VSMC 299

6.1.4. Therapeutic implications of synergism 302

6.1.5. cGI-PDE inhibition in other tissues 304

Chapter 7. References 309

List of Figures

Fig. 1.1.	*Signal transduction pathways in platelet activation*	*9*
Fig. 2.1.	*Labelling of washed rabbit platelets with [^{3}H]guanine and [^{3}H]adenine*	*113*
Fig. 2.2.	*Representative two-dimensional t.l.c. separation of nucleotides potentially labelled after incubation of cultured VSMC with [^{3}H]hypoxanthine*	*115*
Fig. 2.3.	*Representative standard curve for radioimmunoassay of cAMP*	*117*
Fig. 2.4.	*Representative standard curve for radioimmunoassay of cGMP*	*119*
Fig. 2.5.	*Effects of pre-load tension on the ability of rat aortic smooth muscle to contract in response to 30 mM KCl*	*121*
Fig. 3.1.	*Inhibition by SNP of PAF-induced platelet aggregation and [^{14}C]5-HT release; synergism between SNP and PGE_1 and reversal of the effects of PGE_1 by DDA*	*161*
Fig. 3.2.	*Effects of different concentrations of SNP on platelet aggregation, [^{3}H]cAMP and [^{3}H]cGMP in the absence and presence of DDA and PGE_1*	*163*
Fig. 3.3.	*Time course of the SNP-induced accumulation of [^{3}H]cGMP and [^{3}H]cAMP in rabbit platelets*	*167*
Fig. 3.4.	*Relationships between the increases in [^{3}H]cGMP and [^{3}H]cAMP in rabbit platelets caused by addition of SNP in the absence and presence of PGE_1*	*169*
Fig. 3.5.	*Effects of SIN-1 on release of rabbit platelet [^{14}C]5-HT and on platelet [^{3}H]cAMP and [^{3}H]cGMP; synergism between SIN-1 and PGE_1 and reversal of the effects of PGE_1 by DDA*	*171*
Fig. 3.6.	*Inhibition by hemoglobin of the increases in [^{3}H]cGMP and [^{3}H]cAMP induced by SNP in the absence or presence of PGE_1*	*173*
Fig. 3.7.	*Effects of cilostamide and PGE_1 on [^{3}H]cAMP in rabbit platelets*	*175*

Fig. 3.8. *Effects of SNP and M&B 22,948 on $[^3H]$cGMP and $[^3H]$cAMP in rabbit platelets* 177

Fig. 3.9. *Relationship between the increases in platelet $[^3H]$cGMP and $[^3H]$cAMP caused by SNP in the absence and presence of M&B 22,948* 179

Fig. 3.10. *Effects of M&B 22,948 on PGE_1-induced increases in $[^3H]$cAMP in rabbit platelets* 181

Fig. 4.1. *Effect of SNP on isoprenaline-induced relaxation of rat aortic rings precontracted by 100 nM phenylephrine* 220

Fig. 4.2. *Effect of SIN-1 on the isoproterenol-induced relaxation of rat aortic rings precontracted by 100 nM phenylephrine* 222

Fig. 4.3. *Effects of nitrovasodilators and of cAMP phosphodiesterase inhibitors on the relaxation by 96 nM isoprenaline of rat aortic rings precontracted by 100 nM phenylephrine* 224

Fig. 4.4. *Effect of cilostamide on isoproterenol-induced relaxation of rat aortic rings precontracted by 100 nM phenylephrine* 226

Fig. 4.5. *Effect of Ro 20-1724 on isoproterenol-induced relaxation of rat aortic rings precontracted with 100 nM phenylephrine* 228

Fig. 4.6. *Effects of isoproterenol and SNP on the contraction of rat aortic smooth muscle by phenylephrine* 230

Fig. 4.7. *Effect of SNP on the ability of isoproterenol to inhibit phenylephrine-induced contractions of rat aortic rings* 232

Fig. 4.8. *Effect of SIN-1 on the ability of isoproterenol to inhibit phenylephrine-induced contractions of rat aortic rings* 234

Fig. 4.9. *Effects of nitrovasodilators and of cAMP phosphodiesterase inhibitors on the ability of 100 nM isoproterenol to inhibit the contraction of rat aortic rings by 100 nM phenylephrine* 236

Fig. 4.10. *Effect of cilostamide on the isoproterenol-induced inhibition of the contraction of rat aortic rings by phenylephrine* 238

List of Figures (Continued)

Fig. 4.11. Effects of Ro 20-1724 on the ability of isoproterenol to inhibit phenylephine-induced contractions of rat aortic rings 240

Fig. 4.12. Effects of SNP and isoproterenol on cyclic nucleotide levels in rat aortic smooth muscle 242

Fig. 5.1. Metabolic conversion of hypoxanthine to ATP and GTP 269

Fig. 5.2. Time course of [^{3}H]hypoxanthine uptake by cultured rat aortic vascular smooth muscle 270

Fig. 5.3. Effects of different concentrations of isoproterenol on the [^{3}H]cAMP level of cultured VSMC 272

Fig. 5.4. Time course of isoproterenol-induced increases in [^{3}H]cAMP in cultured VSMC 274

Fig. 5.5. SNP-induced increases in cGMP in cultured VSMC (W1) 276

Fig. 5.6. Time course of the increases in cGMP caused by SNP in VSMC (W1) 278

Fig. 5.7. Concentration-dependence of adenosine-induced increases in [^{3}H]cAMP in cultured VSMC (W2) 280

Fig. 5.8. Time dependence of the adenosine-induced increases in [^{3}H]cAMP in prelabelled cultured VSMC (W2) 282

List of Tables

Table 1.1. Agonists capable of affecting VSM tone 31

Table 2.1. Digitonin permeabilization of washed rabbit platelets 111

Table 2.2. R_f values of nucleotides in the two-dimensional t.l.c. purification method used to separate ATP and GTP from acidified extracts of cultured vascular smooth muscle cells [from Randerath and Randerath (1964)] 112

Table 3.1. Purity of [^{3}H]cAMP and [^{3}H]cGMP isolated from prelabelled rabbit platelets by the two column procedure 183

Table 3.2. Comparison of values obtained for platelet cyclic nucleotides using prelabelling assays and radio-immunoassays 186

Table 3.3. Synergism between SNP and adenosine with respect to increases in platelet [^{3}H]cAMP but not [^{3}H]cGMP 188

Table 3.4. Effects of hemoglobin on the synergistic interactions between nitrovasodilators and activators of adenylyl cyclase 189

Table 3.5. Comparison of the effects of SNP and cilostamide on platelet cyclic [^{3}H]nucleotides 191

Table 3.6. Effects of SNP and M&B 22,948 on [^{3}H]cGMP and [^{3}H]cAMP in rabbit platelets 192

Table 3.7. Synergistic effects of SNP and PGE_1 on rat platelet aggregation 194

Table 3.8. Effects of SNP, cilostamide and PGE_1 on cyclic [^{3}H]nucleotide levels in rat platelets 195

Table 4.1. Effects of low concentrations of nitrovasodilators and of inhibitors of cAMP phosphodiesterase on the ability of isoproterenol to relax rat aortic rings previously contracted by 100 nM phenylephrine 244

Table 4.2. Effects of low concentrations of nitrovasodilators and of inhibitors of cAMP phosphodiesterase on the ability of isoproterenol to inhibit the contraction of rat aortic rings by 100 nM phenylephrine 245

List of Tables (Continued)

Table 4.3. Effects of 50 nM SNP and 500 nM isoproterenol on cyclic nucleotide levels in rat aortic smooth muscle 246

Table 5.1. Effects of isoproterenol or adenosine on the level of [^{3}H]cAMP in different cultured VSMC lines 284

Table 5.2. Pharmacological characteristics of rat aortic cultured VSMC 285

Table 5.3. Purity of [^{3}H]cAMP and [^{3}H]cGMP isolated from prelabelled cultured vascular smooth muscle cells by the two column method 286

Table 5.4. Comparison of VSMC cGMP and cAMP content as determined using a prelabelling assay and radioimmunoassays 287

Table 5.5 Effects of different vasodilators on the levels of [^{3}H]cAMP and [^{3}H]cGMP in two distinct cultured VSMC lines 290

Table 5.6. Effects of SNP, cilostamide and Ro 20-1724 on the increases in [^{3}H]cAMP caused by isoproterenol in cultured VSMC (W1) 291

Table 5.7. Effects of SNP, cilostamide and Ro 20-1724 on the increases in [^{3}H]cAMP induced by adenosine in cultured VSMC (W2) 292

LIST OF ABBREVIATIONS

AA	arachidonic acid
AAS	antibiotic-antimycotic solution
ABP	actin-binding protein
ACD	acid citrate dextrose
ACS	aqueous counting scintillant
AD	adenosine
ADP	adenosine 5'-diphosphate
AMP	adenosine 5'-monophosphate
ATP	adenosine 5'-triphosphate
8-BrcGMP	8-bromo guanosine cyclic 3',5'-monophosphate
BSA	bovine serum albumin
$[Ca^{2+}{}_i]$	intracellular calcium concentration
cAMP	adenosine cyclic 3',5'-monophosphate
cGMP	guanosine cyclic 3',5'-monophosphate
DAG	*sn*-1,2-diacylglycerol
DDA	2',5'-dideoxyadenosine
DMEM	Dulbecco's Modified Eagle's Medium
d.p.m.	disintegrations per minute
EDTA	ethylenediamine tetraacetic acid
EGTA	ethyleneglycol-bis-(β-aminoethyl ether)*N*,*N'*-tetraacetic acid
FCS	Fetal calf serum
G-protein	guanine nucleotide binding protein
g_{av}	g force at the mean radius of the centrifuge tube

g_{max}	maximum g force exerted on the tube
GDP	guanosine 5'-diphosphate
GMP	guanosine 5'-monophosphate
GTP	guanosine 5'-triphosphate
HBSS	Hank's buffered salt solution
HEPES	*N*-2-hydroxyethylpiperazine-*N'*-2-ethanesulphonic acid
5-HT	serotonin
IBMX	3-isobutyl-1-methylxanthine
IC_{50}	concentration causing 50% inhibition
IDP	inosine 5'-monophosphate
IMP	inosine 5'-diphosphate
ITP	inosine 5'-triphosphate
PA	phosphatidic acid
PAF	platelet activating factor
PDE	cyclic 3',5'-nucleotide phosphodiesterase
PEI	polyethyleneimine
PKC	protein kinase C
PGE_1	prostaglandin E_1
PGD_2	prostaglandin D_2
PGI_2	prostacyclin
PI	phosphatidylinositol
PIP	phosphatidylinositol 4-phosphate
PIP_2	phosphatidylinositol 4,5-bisphosphate
PIPES	piperazine-*N*,*N'*-bis(2-ethanesulphonic acid)
PLA_2	phospholipase A_2

PRP	platelet-rich plasma
PSS	physiological salt solution
R_f	ratio of the distances travelled by a compound the solvent in thin layer chromatography
Ser	Serine
S.E.M.	standard error of the mean
S.E.Q.	standard error of the quotient
SNP	sodium nitroprusside
SIN-1	3-morpholinosydnonimine
t.l.c.	thin layer chromatography
TCA	trichloroacetic acid
Thr	Threonine
TXA_2	thromboxane A_2
v/v	volume/volume
VSM	vascular smooth muscle
VSMC	vascular smooth muscle cells
w/v	weight/volume
XTP	xanthine 5'-triphosphate
XDP	xanthine 5'-diphosphate
XMP	xanthine 5'-monophosphate

Chapter 1. Introduction

1.1. *Platelet structure*

Blood platelets are small anucleate discoid cells. These cells have an extensively invaginating plasma membrane that forms what is referred to as a surface-connected open canalicular system (reviewed by White, 1987). Platelets have many plasma membrane-associated glycoproteins, most of which are involved either directly or indirectly in adhesion to the subendothelium or the platelet aggregation response (reviewed by Baumgartner and Muggli, 1976). These have been designated GPIa, Ib, Ic, IIa, IIb, IIIa, IIIb, IV, V and IX in order of decreasing molecular mass (Clemetson and Lüscher, 1988). Immediately beneath the plasma membrane lies the platelet membrane cytoskeleton (reviewed by Fox, 1987). This cytoskeleton is composed of short actin filaments cross-linked by actin-binding protein (ABP) which is also bound to GPIb. Other proteins are associated with the platelet cytoskeleton and participate in linking actin filaments to the membrane (talin, vinculin, α-actinin and spectrin). Additional proteins are involved in the contractile response of the platelet (myosin, tropomyosin and caldesmon) (reviewed by Fox, 1987). Platelets also contain an circumferential band of microtubules which run around the perimeter of the platelet (reviewed by White, 1987). This equatorial band of microtubules is involved in maintaining the platelet discoid shape.

Platelets possess an extensive intracellular membrane network. This structure, the dense tubular system, is involved in the control of intracellular Ca^{2+} concentration ($[Ca^{2+}{}_i]$) (reviewed by Zucker and Nachmias, 1985; Kroll and Schafer, 1989; Siess, 1989). Platelets also contain a large number of mitochondria, glycogen granules and three distinct types of secretory granule. Platelet dense granules contain ADP, ATP and 5-HT, whereas the α-granules contain proteins, including fibrinogen, fibronectin, thrombospondin, platelet factor 4, β-thromboglobulin and platelet derived growth factor. The third type of granule contains lysosomal enzymes (i.e. acid hydrolases) (reviewed by Zucker and Nachmias, 1985; Kroll and Schafer, 1989; Siess, 1989).

1.2. *Platelet physiology and pharmacology*

Blood platelets play an integral role in thrombosis and hemostasis. By adhering to sites of vessel wall damage, these cells form a physical barrier. Also, when activated they release factors that stimulate the deposition of other platelets, as well as the recruitment of inflammatory and other cells necessary for vessel wall repair (reviewed by Baumgartner and Muggli, 1976). The subendothelium may be exposed as a result of damage or the formation of gaps attributable

to endothelial cell retraction. The proteins present in the subendothelium with which platelets interact are collagen and von Willebrand Factor (vWF) (reviewed by Baumgartner and Muggli, 1976; Siess, 1989). There are at least four types of collagen present in the subendothelial space (type I, III, IV and V) but only types I, III and IV can promote adhesion (reviewed by Siess, 1989). Results of recent work suggest that the receptor for collagen is GP Ia and that for vWF is GPIb (reviewed by Siess, 1989). GP Ib is composed of two distinct subunits (GP Ibα and GP Ibβ) (reviewed by Siess, 1989). In addition to their ability to promote platelet adhesion, collagen and vWF at sufficiently high concentrations will promote release of platelet granular contents and cause platelet aggregation.

There are many other physiological agents which are known to cause platelet aggregation and numerous other agonistic effects in other cells, particularly smooth muscle. These agents include thrombin, platelet-activating factor (PAF), ADP, 5-HT, [8-arginine]vasopressin and epinephrine. The first effect of many aggregating agents (but not epinephrine) is the shape change reaction. During this process the platelet becomes more spherical, pseudopods form and GP IIb/IIIa, which has been identified as the fibrinogen receptor, is exposed (reviewed by Zucker and Nachmias, 1985; Kroll and Schafer, 1989). The shape change reaction involves major restructuring of the platelet cytoskeleton (reviewed by Fox, 1987). One of the first steps in this process is the phosphorylation of the myosin light chain (MLC), by the

Ca^{2+}/calmodulin-dependent myosin light chain kinase (MLCK) (Daniel *et al.*, 1984). This phosphorylation event allows inactive myosin to interact with actin and thus activate the myosin ATPase activity and the contractile response (Adelstein and Conti, 1975). The contractile action of platelet actomyosin brings about granule centralization which is followed by fusion of the granules with the open canalicular system, so allowing the granular contents to be expelled from the platelets (reviewed by White, 1987). Many of the actin-associated proteins are also redristributed during platelet aggregation. Tropomyosin, caldesmon and ABP all become concentrated in the pseudopodia (reviewed by Fox, 1987).

The release reaction is necessary for the establishment of irreversible aggregation (reviewed by Zucker and Nachmias, 1985; Kroll and Schafer, 1989; Siess, 1989). Thus, thrombospondin, an α-granule product released from stimulated platelets, binds to activated platelets and is required for irreversible aggregation. Also, ADP and 5-HT released from the platelet dense granules, and fibrinogen and fibronectin released from the α-granules, are involved in the recruitment of other platelets to the platelet aggregate (reviewed by Zucker and Nachmias, 1985).

Although aggregating agents cause platelet activation by interacting with specific platelet membrane receptors, the identity of some of these receptors (e.g. for thrombin) is still not clear. Also,

only some of the mechanisms by which platelets are activated by these agents have been elucidated. In this section, I will introduce the two aggregating agents that I used in my work (thrombin and PAF). Whereas thrombin causes platelet aggregation in most species, PAF is a particularly potent activator of rabbit platelets.

1.2.1. *Thrombin*

Thrombin is a serine protease formed from circulating prothrombin. Protease inhibitors, such as diisopropyl fluorophosphate (DFP), have been shown to inhibit thrombin-induced platelet aggregation (reviewed by Majerus *et al.*, 1976). Platelet responses to low concentrations of thrombin are dependent on the release of arachidonic acid and its metabolism to TXA_2 (see Section 1.3.4.). However, higher concentrations of thrombin cause full platelet aggregation and release of granule constituents in the total absence of TXA_2 or ADP (reviewed by Zucker and Nachmias; Kroll and Schafer, 1989; Siess, 1989). Binding experiments have suggested that there may be at least two types of thrombin receptors on platelet membranes (Harmon and Jamieson, 1986). The putative vWF receptor, GP Ibα, has also been proposed to be a thrombin receptor (Larsen and Simon, 1981). This conclusion was based on the formation of a 200 kDa complex following cross-linking of thrombin to platelet membranes. In identical experiments using platelets lacking GP Ib (Bernard-Soulier patients), no such complex was formed. However, the response of Bernard-Soulier

platelets to maximum concentrations of thrombin are virtually normal (George *et al.*, 1981). Also, since platelet aggregation by thrombin involves the activation of platelet phospholipase C (see Section 1.3.3.), and this process is GTP-dependent (Hrbolich *et al*, 1987), the receptor mediating the effects of thrombin will likely have seven membrane spanning domains (see Section 1.10.1.), which GP Ibα does not (Lopez *et al.*, 1988). Thus, another membrane protein may be involved in mediating the effects of thrombin. The relationship between binding of thrombin to GP Ibα and the interaction of thrombin with the receptor that is coupled to activation of platelet phospholipase C is not clear.

1.2.2. *Platelet activating factor*

Platelet activating factor (PAF, 1-O-alkyl-2-acetyl-*sn*-glycero-3-phosphocholine) (Demopoulos *et al.*, 1979), is one of the most potent aggregating agents known. Although PAF causes full aggregation of platelets from most species (i.e shape change, aggregation and release), mouse and rat platelets do not have PAF receptors (reviewed by Siess, 1989). Upon platelet activation, PAF is synthesized and released from rabbit platelets (Chignard *et al.*, 1980). Human platelets do not make appreciable amounts of PAF, but PAF is also synthesized and released from activated neutrophils, basophils, macrophages, mast cells and endothelial cells (reviewed by Braquet *et al.*, 1987). Although PAF alone will cause complete aggregation and

release of granule constituents from rabbit platelets (Cazenave *et al.*, 1979), irreversible aggregation of human platelets by PAF requires the formation of TXA_2 (Section 1.3.4.) and the release of ADP (Osterman *et al.*, 1983). PAF receptors have been studied in both human and rabbit platelets (reviewed by Siess, 1989). Rabbit platelets have been shown to have high affinity binding sites (K_d=1.4 nM) for PAF (Hwang *et al.*, 1983). GTP inhibited PAF binding and PAF stimulated a membrane-bound GTPase activity (Hwang *et al.*, 1986). Thus, PAF receptors must be coupled to effector enzymes through one or more GTP-binding proteins.

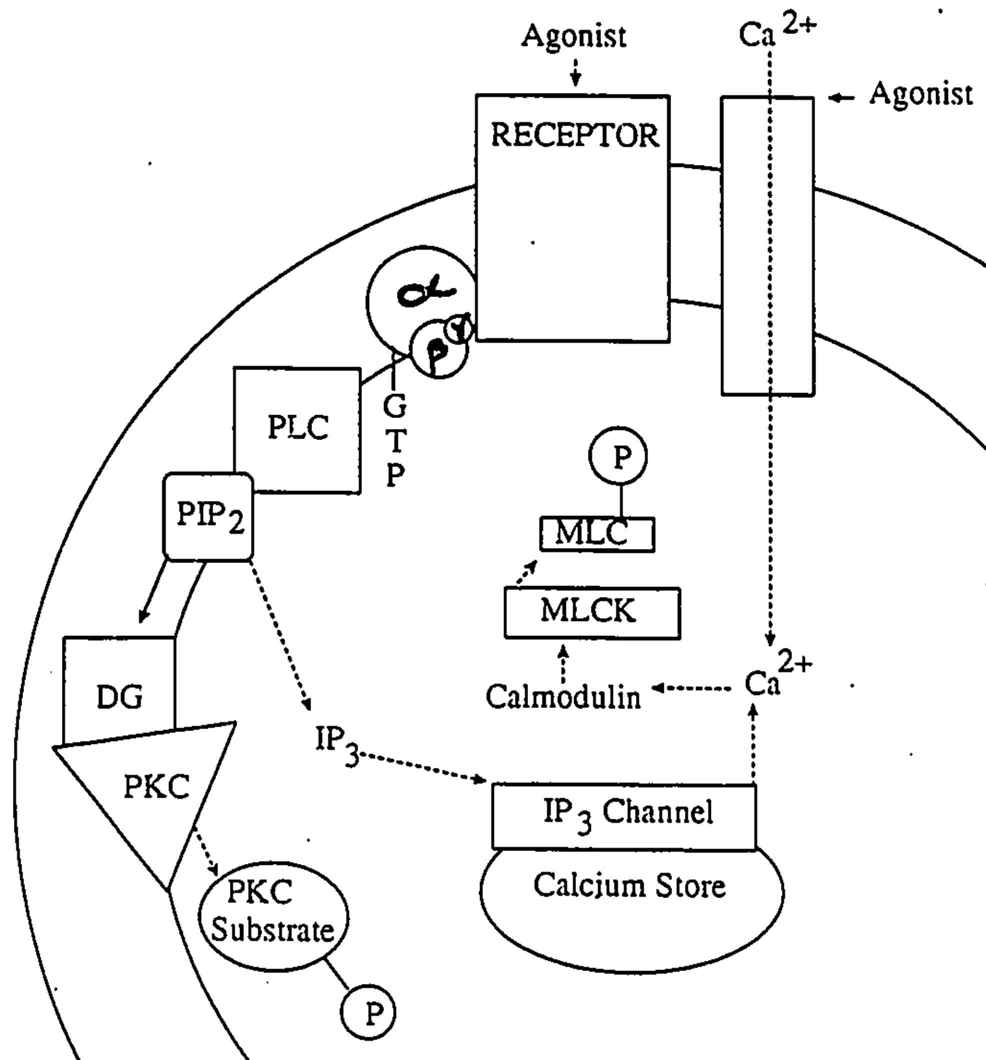
1.3. *Signal transduction pathways in platelet activation*

1.3.1. *General considerations*

Platelet aggregation can be initiated by a variety of different converging pathways (Fig. 1.1). Most aggregating agents act by increasing intracellular Ca^{2+} ($[Ca^{2+}{}_i]$) (Rink and Sage, 1990). Increases in platelet $[Ca^{2+}{}_i]$ can be stimulated by at least two different pathways (Fig. 1.1.). Thus, agonist-induced activation of platelet phospholipase C (PLC) stimulates the hydrolysis of phosphatidyl

Fig. 1.1. Signal transduction pathways in platelet activation

The diagram depicts the major signal transduction pathways which are known to be involved in agonist-mediated platelet activation.


Agonist
Ca 2+
Agonist
RECEPTOR
G
T
P
PLC
PIP2
P
MLC
MLCK
DG
Calmodulin
Ca 2+
IP3
PKC
IP3 Channel
Calcium Store
PKC
Substrate
P

inositol 4,5-bisphosphate (PIP_2) with the formation of inositol 1,4,5-trisphosphate ($Ins(1,4,5)P_3$), which is known to cause release of Ca^{2+} from intracellular stores, including the dense tubular system (reviewed by Haslam, 1987; Berridge, 1987) (Fig. 1.1). Although activation of platelet PLC is GTP-dependent, the GTP-binding protein (G-protein) involved, which has been designated G_p, is still unclear (reviewed by Haslam, 1987; Brass, 1989). Platelets also have receptor-operated Ca^{2+} channels that allow Ca^{2+} entry, notably in response to stimulation by ADP (reviewed by Rink and Sage, 1990) (Fig. 1.1.). Ca^{2+} acts at many points in the signal transduction pathways involved in platelet aggregation. A Ca^{2+}/calmodulin complex has been shown to activate platelet MLCK (reviewed by Haslam, 1987; Siess, 1989) (Fig. 1.1.). Also, Ca^{2+} facilitates the activation of platelet protein kinase C (PKC). Although high concentrations of Ca^{2+} alone can activate PKC, the 1,2-diacylglycerol (DAG) formed as a result of the hydrolysis of phosphoinositides by PLC reduces the Ca^{2+} concentration required for activation of PKC to physiological levels (reviewed by Nishizuka, 1984). Relatively little is known about the role(s) of PKC substrates in platelet function but phorbol esters, which are pharmacological activators of PKC, do cause platelet aggregation and release of granule constituents (reviewed by Haslam, 1987; Siess, 1989). The Ca^{2+}-mediated activation of MLCK and the Ca^{2+}-independent stimulation of PKC can act in concert to bring about secretion of granule constituents and with it irreversible platelet aggregation (Nishizuka, 1984). Activation of platelet

phospholipase A_2 (PLA_2) releases arachidonic acid (AA). PLA_2 can be activated either by receptor-G-protein interactions (Silk *et al.*, 1989) or by an increase in [$Ca^{2+}{}_i$] (reviewed by Haslam, 1987; Siess, 1989). Thromboxane A_2 (TXA_2) is formed from AA by the sequential actions of cyclooxygenase and thromboxane synthase and is a potent activator of platelet PLC by a receptor-G-protein dependent mechanism (reviewed by Brass, 1989; Siess, 1989). Since aggregating agents cause increases in [$Ca^{2+}{}_i$] and these increases in Ca^{2+} concentration can in turn activate PLA_2, platelets possess a mechanism whereby amplification of the initial aggregating stimulus can occur. Epinephrine, which greatly potentiates the effects of other aggregating agents and under some conditions causes aggregation alone, may exert important effects through activation of phospholipase A_2 (Limbird, 1988)

1.3.2. *GTP-binding proteins*

There is increasing evidence that many of the receptors involved in signal transduction in platelets are coupled to effector enzymes by GTP-binding proteins (G-proteins). A class of G-proteins that has been extensively studied are the heterotrimeric G-proteins which contain α, β and γ subunits (reviewed by Freissmuth *et al.*, 1989; Birnbaumer, 1990). Initially, the GTP-binding subunits, $G_{s\alpha}$ and $G_{i\alpha}$, were identified in platelets on the basis of their specific ADP-ribosylation by cholera and pertussis toxins, respectively

(reviewed by Freissmuth *et al.*, 1989). Using molecular biological approaches it has been shown that there exist at least 9 and possibly 12-15 distinct G-protein α-subunits (Birnbaumer, 1990). In addition to G_s and three forms of G_i (G_{i1}, G_{i2} and G_{i3}), platelets possess a novel G-protein of unknown function, G_z (Casey *et al.*, 1990). Many lower molecular mass GTP-binding proteins have also been identified (Hall, 1990). Recently, it has been suggested that these smaller G-proteins may operate in concert with the larger G-proteins to control effector function (Yatani *et al.*, 1990). Platelets contain *ras*, *ral*, *rac*, *rap* and other low molecular mass GTP-binding proteins (Bhullar and Haslam, 1988; Polakis *et al.*, 1989; Lapetina, 1990).

1.3.3. *Phospholipase C*

Since the initial discovery of Hokin and Hokin (1955) that agonists could alter the incorporation of phosphate into membrane phospholipid, there has been considerable interest in the role of membrane phospholipid in cellular signalling. More recently, the focus has been on the hydrolysis of polyphosphoinositides by phospholipase C. In platelets this is due in part to the early studies of Rittenhouse-Simmons (1979), in which thrombin was shown to cause a rapid increase in the amount of 1,2-diacylglycerol (DAG) in platelets. DAG is a relatively short-lived compound in platelet membranes and is quickly converted to phosphatidic acid (PA) by diglyceride kinase. It is now

well established that in platelets most of the DAG/PA formed in response to an agonist is the result of the action of PLC on phosphatidyl inositol-4-phosphate (PIP) and phosphatidylinositol 4,5-bisphosphate (PIP_2) (reviewed in Siess, 1989; Rhee *et al.*, 1989). Many platelet aggregating agents, including thrombin, vasopressin and PAF, have been shown to cause transient increases in DAG/PA formation in platelets (reviewed by Siess, 1989). Also, platelet aggregation by agents that activate platelet PLC have been associated with transient decreases in the levels of platelet PIP_2 (reviewed by Siess, 1989). Some agonists induce platelet aggregation without the activation of platelet PLC (e.g. epinephrine or ADP) (reviewed by Siess, 1989).

The products of the PLC reaction are DAG and inositol phosphates (reviewed by Berridge, 1987). DAG is an important regulator of the activity of a Ca^{2+}/phosphatidylserine-activated protein kinase (PKC) (see Section 1.3.5.). The type of inositol phosphate formed will necessarily depend on the nature of the phosphoinositide substrate. Polyphosphoinositides, which make up only a small percentage of the total membrane phospholipids, are the preferred substrates. Thus, the inositol phosphate formed will be inositol 1,4-bisphosphate ($Ins(1,4)P_2$) and inositol 1,4,5-trisphosphate ($Ins(1,4,5)P_3$) when PIP and PIP_2 are the substrates, respectively. $Ins(1,4,5)P_3$ has been shown to stimulate the release of Ca^{2+} from internal stores in many cells (reviewed by Berridge, 1987). Formation of $Ins(1,4,5)P_3$

following incubation of intact human platelets with many aggregating agents, including thrombin and PAF, is well-established and has been extensively studied (reviewed by Haslam, 1987; Siess, 1989). The formation of inositol cyclic 1,2-phosphates following activation of platelet PLC has been reported (Majerus *et al.*, 1990). However, the role(s) of these inositol cyclic phosphates in cell signalling is not yet clear.

In platelets, there are soluble and particulate isozymes of PLC. The cytosolic isozyme (PLC_{γ}) is activated by increases in $[Ca^{2+}{}_{i}]$ (reviewed by Rhee *et al.*, 1989). It is generally accepted that the receptor-mediated activation of the particulate form(s) of platelet PLC mediates the effects of activators of platelet aggregation (reviewed by Haslam, 1987; Rhee *et al.*, 1989). Also, activation of the platelet particulate PLC has been shown to be GTP-dependent (Hrbolich *et al.*, 1987). However, the identity of the G-protein(s) that regulate PLC activity in platelets is uncertain (reviewed by Haslam, 1987; Brass, 1989; Siess, 1989).

1.3.4. *Phospholipase A_2*

In addition to the receptor-mediated hydrolysis of phosphoinositides by PLC, activation of phospholipase A_2 (PLA_2) is often observed during cellular activation. In platelets, PLA_2 cleaves

phosphatidylcholine (PC) and phosphatidylethanolamine (PE) at the C-2 position to release arachidonic acid (AA) (reviewed by Haslam, 1987; Kroll and Schafer, 1989; Siess, 1989). The AA released by the action of PLA_2 on these phospholipids is rapidly converted to a large number of eicosanoids. In platelets, one of the most important products formed is thromboxane A_2 (TXA_2). The conversion of AA into TXA_2 is catalyzed by the sequential action of both cyclo-oxygenase and thromboxane synthase. TXA_2 is a potent activator of platelet PLC, and when released by activated platelets, participates in the aggregation and secretion responses (reviewed by Zucker and Nachmias, 1985; Kroll and Schafer, 1989; Siess, 1989). AA is also converted to 12-monohydroperoxy and 12-monohydroxy derivatives by 12-lipoxygenase (reviewed by Kroll and Schafer, 1989; Siess, 1989). Platelet PLA_2 can be activated by a G-protein-dependent mechanism and by increases in intracellular Ca^{2+} (Ca^{2+}_i) (Silk *et al.*, 1989).

1.3.5. *Protein kinase C*

Certain serine-threonine protein kinases, the protein kinase C (PKC) family are activated by phosphatidylserine and Ca^{2+} (Kikkawa *et al.*, 1989). During platelet aggregation, a DAG-dependent translocation of PKC to the plasma membrane occurs and DAG reduces the concentration of Ca^{2+} required for enzyme activity to physiological levels (reviewed by Siess, 1989; Kikkawa *et al.*, 1989). Much of the information available concerning PKC activity comes from studies in

which platelets were treated with phorbol esters (e.g. phorbol 12-myristate 13-acetate, PMA), which are pharmacological activators of PKC and cause platelet aggregation. Platelet PKC can also be activated by proteolysis (reviewed by Kikkawa *et al.*, 1989). In platelets, the major substrate for PKC is a 47 kDa cytosolic protein (Imoaka *et al.*, 1983). This platelet protein was recently cloned and has been called pleckstrin (Tyers *et al.*, 1988). Phosphorylation of pleckstrin is observed in the presence of stimuli that cause the platelet release reaction (reviewed by Haslam, 1987; Kroll and Shafer, 1989; Siess, 1989), but its function remains elusive. Other platelet substrates of PKC that may be of importance in signal transduction include the G-proteins, G_z and possibly G_i (Katada *et al.*, 1985; Williams *et al.*, 1987; Carlson *et al.*, 1988). The PKC-induced phosphorylation of G_i can inhibit its ability to modulate adenylyl cyclase activity.

1.4. *Role of Ca^{2+} in platelet aggregation*

Most aggregating agents (with the exception of epinephrine) bring about transient increases in the level of intracellular Ca^{2+} ($[Ca^{2+}{}_i]$) as measured by fluorescent probes, such as quin2 and fura-2 (reviewed by Haslam, 1987; Kroll and Schafer, 1989; Siess, 1989). Measurement of $[Ca^{2+}{}_i]$ in unstimulated platelets using fura-2 gives a value equal to or even slightly below 100 nM (reviewed by Rink and Sage, 1990). Upon platelet stimulation, the $[Ca^{2+}{}_i]$ can

increase to values as high as 1 μM. Initial attempts to measure the Ca^{2+} transients that occurred during agonist-induced platelet activation were impaired by the extensive Ca^{2+} buffering capacity of the fluorescent indicators used in these studies (e.g. quin-2) (reviewed by Haslam, 1987; Rink and Sage, 1990). It is generally accepted that agonist-induced increases in $[Ca^{2+}{}_i]$ play an important role in platelet responses to certain agonists. However, there are some indications that platelet reactions (shape-change, release of granular constituents and MLC phosphorylation) can occur without any measurable increase in $[Ca^{2+}{}_i]$ (reviewed by Haslam, 1987; Siess, 1989). Some of these effects may be mediated by the activation of PKC. How do increases in platelet $[Ca^{2+}{}_i]$ occur? Ca^{2+} can be mobilized from intracellular storage sites (the dense tubular system) by activation of the phosphoinositide/Ca^{2+} mobilization pathway (see Section 1.3.3.). The free Ca^{2+} in the dense tubular system has been estimated at several hundred micromolar (Sage and Rink, 1989). Since the extracellular Ca^{2+} concentration is three orders of magnitude greater than $[Ca^{2+}{}_i]$, Ca^{2+} ions can also enter from the extracellular medium.

1.4.1 *Ca^{2+} influx*

Most cells have both voltage-operated Ca^{2+} channels and receptor-operated Ca^{2+} channels. The most important voltage-operated Ca^{2+} channels in many cells are blocked by dihydropyridines (DHP),

have relatively high conductances and inactivate very slowly (L-type Ca^{2+} channels). However, platelets do not possess L-type Ca^{2+} channels (Rink and Sage, 1986). Thus, incubation of platelets with depolarizing concentrations of KCl does not cause a significant increase in $[Ca^{2+}_i]$ (Rink and Sage, 1986). Moreover, membrane depolarization was shown to inhibit thrombin-induced platelet aggregation and reduce the influx of Ca^{2+} into these cells (Kovacs et al., 1990). Using patch-clamping techniques, Kawa (1990a) identified T-type Ca^{2+} currents in guinea-pig megakaryocytes. In the latter report, Ca^{2+}-dependent action potentials were recorded when the patch was depolarized beyond -55 mV. The presence of T-type Ca^{2+} channels has not yet been described in platelets. However, voltage-dependent K^+ channels and Ca^{2+}-activated K^+ channels have both been studied in platelets (Maruyama, 1987; Fine *et al.*, 1989) and in megakaryocytes (Kawa, 1990b). In the latter cell, an ADP-induced Ca^{2+} influx was shown to activate the K^+ channel.

ADP has been shown to induce increases in platelet $[Ca^{2+}_i]$ without any measurable delay, whereas delays of 200-300 ms are usually associated with the transient increases in platelet $[Ca^{2+}_i]$ caused by other aggregating agents (Sage *et al.*, 1989; Sage *et al.*, 1990). The Ca^{2+} channel activated by ADP was not affected by changes in membrane potential. A similar channel has also been studied in megakaryocytes by Kawa (1990b). Sage *et al.* (1990) have suggested that the amount of Ca^{2+} that enters the cell via the ADP-coupled

channel is dependent on the amount of Ca^{2+} in the dense tubular system (i.e. related to refilling of internal stores).

1.4.2. *Ca^{2+} release from the dense tubular system*

It is well established that $Ins(1,4,5)P_3$, formed by the action of PLC on PIP_2 (Section 1.3.3.), can stimulate the release of Ca^{2+} from intracellular stores in many cell types (reviewed by Berridge, 1987). $Ins(1,4,5)P_3$ has been shown to release Ca^{2+} from $^{45}Ca^{2+}$-loaded platelet vesicles (Brass and Joseph, 1985; O'Rourke *et al.*, 1987). Also, addition of $Ins(1,4,5)P_3$ to permeabilized platelets induces significant Ca^{2+} mobilization and causes platelet aggregation and the release reaction (O'Rourke *et al.*, 1987). The similarity in the amount of Ca^{2+} released by thrombin or Ca^{2+} ionophore indicates that all the intracellular Ca^{2+} can be released upon platelet activation (Rink *et al.*, 1982). However, the amount released by $Ins(1,4,5)P_3$ is not equivalent to that released by thrombin or ionophore (Authi *et al.*, 1986). A role for inositol 1,2-cyclic 4,5-trisphosphate in mediating intracellular Ca^{2+} mobilization has also been proposed (Majerus *et al.*, 1990).

1.5. *Inhibition of platelet function by cyclic nucleotides*

It is now well established that increases in intracellular cAMP and cGMP inhibit platelet aggregation (Zucker and Nachmias, 1985; Kroll

and Schafer, 1989; Siess, 1989). In early experiments, increases in platelet cGMP that correlated well with aggregation were reported (reviewed by Walter, 1989). However, it was found that the formation of cGMP is a consequence of rather than a cause of platelet aggregation (Haslam *et al.*, 1978; Haslam *et al.*, 1980). The most probable molecular basis for this effect has recently been elucidated. Nitric oxide (NO·) synthase, an enzyme that catalyses the formation of NO· from L-arginine (Palmer *et al.*, 1988) has been shown to be a Ca^{2+}/calmodulin stimulated enzyme (Bredt and Snyder, 1990). Since this enzyme is present in platelets (Radomski *et al.*, 1990a,b), and NO· activates soluble guanylyl cyclase, the increase in Ca^{2+} which occurs upon platelet activation could cause a transient increase in cGMP in these cells. Thus, as initially suggested (Haslam *et al.*, 1978), cGMP formed in this manner may have inhibitory feedback effects (Radomski *et al.*, 1990a).

1.5.1. *cAMP and cGMP-mediated phosphorylation of platelet proteins*

A relatively large number of platelet proteins are phosphorylated when intact platelets are incubated with agents that increase the levels of cAMP or cGMP (Haslam *et al.*, 1978; Fox *et al.*, 1979; Haslam *et al.*, 1980; Waldmann *et al.*, 1987). Thus, treatment of $[^{32}P]P_i$-labelled platelets with agents that increased platelet cAMP levels (PGE_1) increased the amount of $[^{32}P]P_i$ incorporated into 82 kDa, 50 kDa, 49 kDa, 36 kDa, 24 kDa and 22 kDa proteins (Fox e *tal.*

1979; Haslam *et al.*, 1980). The 22 kDa and 24 kDa proteins were membrane-bound and phosphorylation of the latter correlated with sequestration of Ca^{2+} by platelet membranes. Incubation of $[^{32}P]P_i$-labelled platelets with agents that increase cGMP levels (SNP) cause phosphorylation of these same platelet proteins. However, the level of phosphorylation of both the 24 kDa and the 22 kDa were markedly less when SNP was used compared to PGE_1. This may reflect the fact that SNP caused only small increases in cAMP in these experiments (Haslam *et al.*, 1980). Consistent with this, 8-BrcGMP, which does not increase cAMP, did not induce the phosphorylation of the latter proteins (Haslam *et al.*, 1980). Addition of cAMP analogues to these platelets caused phosphorylation of the same proteins as PGE_1. In very similar work, Waldmann *et al.* (1987) reported that PGE_1 increased the phosphorylation of 240 kDa, 68 kDa, 50 kDa, 24 kDa, 22 kDa proteins. Also, a high concentration of SNP increased the $[^{32}P]P_i$ incorporated into 50 kDa, 49 kDa, 24 kDa and 22 kDa proteins. In broken cell preparations, addition of either cAMP or cGMP also causes the phosphorylation of these same proteins. However, in the latter preparations, the actions of both cyclic nucleotides were much less specific (Waldmann *et al.*, 1986).

Recently, the 50 kDa platelet protein that is phosphorylated by both cAMP- and cGMP-dependent mechanisms was purified to homogeneity (Halbrügge and Walter, 1989; Halbrügge *et al.*, 1990). Although this protein migrates with an apparent molecular mass of 50 kDa in its

phosphorylated form, in its non-phosphorylated form the protein has a molecular mass of 46 kDa. Based on an analysis of tryptic fragments of the phosphorylated protein, it was concluded that both the cAMP and cGMP stimulated phosphorylation at a common site. Also, it was concluded that the 49 kDa and 50 kDa proteins identified in the earlier studies were the same protein, at different levels of phosphorylation (Halbrügge and Walter, 1989; Halbrügge *et al.*, 1990). Although these authors speculated that this protein is involved in the cAMP- and cGMP-mediated inhibitions of platelet function, its role is still unknown.

Initial studies had proposed that the 22 kDa protein phosphorylated in response to increases in cAMP (later called thrombolamban) stimulated Ca^{2+}-uptake by the dense tubular system (Käser-Glanzmann *et al.*, 1979). Although there was little evidence to support this hypothesis, it quickly gained acceptance (see Kroll and Schafer, 1989; Siess, 1989). Recently, another role for this protein has been proposed (Lapetina, 1990). Using specific monoclonal antibodies, White *et al.* (1990) have shown that thrombolamban is immunologically identical to *rap 1b*, a member of the *ras* family of low molecular mass GTP-binding proteins, that is phosphorylated by cAMP-PK (reviewed by Lapetina, 1990). It has been suggested that the phosphorylation of *rap 1b*, followed by its translocation to the cytosol, may be responsible for the inhibition of PLC by increases in cAMP (reviewed by Lapetina, 1990). However, this hypothesis is difficult to reconcile with the slow time course of cAMP-mediated

phosphorylation of this protein (Haslam *et al.*, 1980; Siess and Lapetina, 1990).

The 24 kDa protein phosphorylated in platelets in response to incubation with agents that increase cAMP levels, has been identified as the β-subunit of GP Ib (Fox *et al.*, 1987). Moreover, cAMP-mediated phosphorylation of this protein has been correlated with inhibition of collagen-induced actin polymerization (Fox and Berndt, 1989). The absence of an effect of cAMP-mediated phosphorylation of GP Ib on actin polymerization in Bernard-Soulier platelets, which lack GP Ib, was consistent with this (Fox and Berndt, 1989). Although the site that is phosphorylated has been determined (Wardell *et al.*, 1989), the molecular basis of this effect is not clear. The fact that ABP, which cross-links GP Ib (Fox, 1987) is also phosphorylated in response to increases in platelet cAMP may be of some importance in this regard (Cox *et al.*, 1984).

1.5.2 *Effects of cAMP and cGMP on platelet* $[Ca^{2+}{}_i]$

Ca^{2+} ions play a very important role in platelet aggregation (see Section 1.4.) and there are many different mechanisms by which $[Ca^{2+}{}_i]$ can be regulated (reviewed by Kroll and Schafer, 1989; Rink and Sage, 1990). There are several intracellular pools of Ca^{2+} including: 1) the dense tubular system; 2) mitochondria; 3) dense granules; 4) membrane phospholipid-associated Ca^{2+}. Also, Ca^{2+} can enter from the extracellular medium (reviewed by Rink and Sage, 1990).

Given the complexity of [$Ca^{2+}{}_i$] homeostasis in platelets, there are several sites at which the cyclic nucleotides could act to inhibit increases in [$Ca^{2+}{}_i$]. Mechanisms that have been investigated as possible sites of cyclic nucleotide action include: 1) binding of agonists to their receptors; 2) operation of plasma membrane Ca^{2+} channels; 3) polyphosphoinositide hydrolysis by PLC; 4) effects on Ca^{2+} kinetics; 5) effects on the function of the contractile proteins.

1.5.2.1. *Effects on agonist binding*

Increases in platelet cAMP and particularly cGMP inhibit the platelet adhesion reaction (Moake *et al.*, 1981; Radomski *et al.*, 1987b). Although the molecular basis for these effects is not clear, the phosphorylation of ABP (Cox *et al.*, 1984) and GP Ibβ (Fox *et al.*, 1987) are possible sites of action for cAMP. However, these proteins are not phosphorylated in response to cGMP. Also, inhibition of thrombin and vWF binding to GP Ib could result from cAMP-mediated phosphorylation of this glycoprotein (reviewed by Fox, 1987).

1.5.2.2. *Effects on platelet plasma membrane Ca^{2+} channels*

Ca^{2+} can enter activated platelets via receptor-operated Ca^{2+} channels (reviewed by Rink and Sage, 1990). Incubation of platelets with cAMP analogues inhibits thrombin-induced uptake of $^{45}Ca^{2+}$ (Imai *et al.*, 1983). Similar results were obtained when platelets were

incubated with agents that increased cAMP levels (Feinstein *et al.*, 1983). Compounds that increase intracellular cGMP also affect agonist-induced Ca^{2+}-influx (Kawahara *et al.*, 1984; Nakashima *et al.*, 1986; Simon *et al.*, 1988; Morgan and Newby, 1989; Rink and Sage, 1990). In a recent report, Morgan and Newby (1989) showed that agents that increase cGMP may inhibit Ca^{2+} influx components that are unaffected by cAMP. Thus, platelet aggregation responses that are entirely dependent on Ca^{2+} influx may be differentially inhibited by agents that increase either cAMP or cGMP.

1.5.2.3. *Effects on phosphatidylinositol hydrolysis*

Both cAMP and cGMP inhibit thrombin-induced increases in platelet DAG (Rittenhouse-Simmons, 1979; Takai *et al.*, 1981; Imai *et al.*, 1983; Simon *et al.*, 1988). Also, cAMP and cGMP inhibit agonist-induced formation of inositol phosphates (Nakashima *et al.*, 1986; Simon *et al.*, 1988). Although both cyclic nucleotides also inhibited the formation of inositol phosphates induced by direct G-protein activation of PLC (Haslam *et al.*, 1988; Deana *et al.*, 1989; Yada *et al.*, 1989), they were less potent inhibitors of the effects of guanine nucleotides. The molecular basis for the effects of the cyclic nucleotides on the G-protein-activated PLC is not clear, but in the case of cAMP may well be related to a phosphorylation event (Yada *et al.*, 1989). Lapetina (1990) has proposed that the substrate phosphorylated in response to cAMP that regulates PLC may be rap 1B.

1.5.2.4. *Effects of cyclic nucleotides on Ca^{2+} kinetics*

$[Ca^{2+}{}_i]$ can be reduced by either active transport of the ions out of the platelet, or by the sequestration of Ca^{2+} by the dense tubular system. There are many reports of cAMP- and cGMP-mediated effects on Ca^{2+} transport by isolated platelet membrane vesicles. Agents that increase cAMP in intact cells increases Ca^{2+} uptake by vesicles isolated from treated platelets (Fox *et al*, 1979; Haslam *et al.*, 1980; Le Peuch *et al.*, 1981). In addition, cAMP has been reported to stimulate Ca^{2+} sequestration by vesicles from untreated platelets (Takai *et al.*, 1981; Enouf *et al.*, 1987; Hettasch and LeBrun, 1987; Adunyah and Dean, 1987). Also, this increase in Ca^{2+} uptake has been correlated to an increase in Ca^{2+} ATPase activity (Adunyah and Dean, 1987). Since cAMP-PK inhibitors reversed the effect of cAMP, it has been suggested that a cAMP-mediated phosphorylation event is responsible for the increased Ca^{2+} uptake (Hettasch and LeBrun, 1987). Recent studies have shown that ATP-dependent Ca^{2+} uptake is exclusively present in the intracellular platelet membranes (Hack *et al.*, 1986). Thus, the cAMP-mediated effects are presumably attributable to an effect on the membranes of the dense tubular system. Although earlier studies had implicated the phosphorylation of 22 kDa or 24 kDa proteins in this process (see Section 1.5.1), this hypothesis now seems less probable (Fox *et al.*, 1987; Siess and Lapetina, 1990). cAMP has also been reported to inhibit $Ins(1,4,5)P_3$-induced Ca^{2+} release from permeabilized platelets

(Enouf *et al*, 1987). In a recent paper, O'Rourke *et al*. (1989) failed to observe an effect of cAMP on either Ca^{2+} uptake or $Ins(1,4,5)P_3$-induced Ca^{2+} release from platelet membrane vesicles. However, most of the evidence indicates that cAMP can inhibit platelet aggregation by inhibiting Ca^{2+}-release.

1.5.2.5. *cAMP and cGMP and the function of contractile proteins*

MLC phosphorylation by MLCK is an early step in the platelet response to most aggregating agents and is thought to be responsible for initiating contractile processes including shape change and centralization of granules. Incubation of platelets with agents that cause increases in cAMP or cGMP levels inhibit this phosphorylation reaction (Haslam *et al*., 1980; Hathaway *et al*., 1981; Cox *et al*., 1984; Nishikawa *et al*., 1984). This effect of cAMP could reflect a cAMP-mediated phosphorylation of MLCK (Conti and Adelstein, 1981). The effects of these cyclic nucleotides thus lead to a reduced interaction of myosin with actin and the platelet cytoskeleton (Cox *et al*., 1984; Nishikawa *et al*., 1986). However, only phosphorylation of MLCK that is not already bound to Ca^{2+}/calmodulin affects enzyme activity (Conti and Adelstein, 1981). Thus, the relationship of this phosphorylation reaction to the inhibition of platelet aggregation is not clear. It may be that the effect of cAMP on $[Ca^{2+}{}_i]$ plays a more important role in inhibiting MLC phosphorylation. The cAMP-mediated phosphorylation of ABP and of the β-subunit of

GP Ib have also been reported (Section 1.5.1.). Thus, increases in cAMP may inhibit several of the changes in cytoskeletal structure that are involved in platelet aggregation (reviewed by Fox, 1987).

1.6. *Structure of large elastic arteries*

Arteries are composed of three histologically distinct layers (reviewed by Rhodin, 1980). The innermost is the intima, composed mostly of endothelial cells and connective tissue. The outermost, the adventia, is composed of connective tissue elements. The medial layer is usually the thickest of the three and it is here that most of the VSMC are found. The VSMC in this area are anchored to elastin fibres and have a spiral orientation around the vessel. The internal elastic lamina, the boundary between the intima and the media, is composed mostly of elastin. Also, the internal elastic lamina has been shown to have fenestrations that may allow communication between the lumen and the VSMC in the media (reviewed by Rhodin, 1980).

VSMC are long and narrow and have a large surface to volume ratio. The cytoplasm of VSMC has a large centrally located nucleus. The plasma membrane has extensive invaginations called caveolae. These caveolae are grouped in rows and lie close to the tubules of the sarcoplasmic reticulum (reviewed by Somlyo, 1980; Gabella, 1984). The sarcoplasmic reticulum tubules are important in VSMC Ca^{2+} handling.

These cells do not have the striations characteristic of skeletal and cardiac muscle. In VSMC, the myosin (thick filaments) and actin (thin filaments) are organized in bundles of microfilaments. Also, these bundles are arranged along the long axis of the VSMC (reviewed by Somlyo, 1980; Gabella, 1984). VSMC also contain intermediate filaments, but the function of these is not yet clear. Dense bodies are attached to the plasma membrane. These stuctures, which contain α-actinin and vinculin, are thought to be important in anchoring actin to the plasma membrane (reviewed by Gabella, 1984). There are also dense bodies present in the cytoplasm of VSMC but the role of these dense bodies in cell function is not clear. Two dense bodies from adjacent VSMC can form cell-to-cell junctions (intermediate junctions). These junctions, which are approximately 50 nm wide, are thought to have a purely mechanical function. Also, gap junctions are formed by the aggregation of channels in the membranes of adjoining VSMC. The proteins involved in this gap junction have recently been under intense study and are now called connexins (reviewed by Beyer *et al.*, 1990). These types of junctions are believed to participate in the propagation of action potentials between the adjacent VSMC (reviewed by Beyer *et al.*, 1990).

1.7. *Vascular smooth muscle physiology and pharmacology*

The VSMC of the vasculature play an important role in the control

of blood flow. This effect is brought about by changes in the contractility of these cells. Since they form a spiral coil around the vessel, a decrease in their length will result in a decrease in the size of the vessel lumen. VSMC either contract or relax, and hence either decrease or increase the luminal volume of blood vessels, in response to many different factors. A partial list of the agonists capable of altering vascular tone is presented in Table 1.1. (Rasmussen, 1987; Bean, 1989; Lincoln, 1989; Lüscher, 1990; Murray, 1990). However, the ability of each of these to increase or decrease the level of contraction will depend on the presence of appropriate receptors or intracellular enzymes, and varies with the source of the VSM.

Table 1.1. *Agonist capable of affecting VSM tone*

Contraction	Relaxation
Phenylephrine	Isoproterenol
Norepinephrine	Epinephrine
Acetylcholine	PGI_2/PGE_1
Histamine	VIP
Serotonin	CGRP
Endothelins	EDRF/NO$^{\cdot}$
$PGF_{2\alpha}$	SNP/SIN-1
Angiotensin	ANP

Physiological regulators of tone may act on VSMC after they are released from nerve endings or the vascular endothelium, or may enter the vessel wall from the circulation. However, the most important site for the synthesis and release of compounds that regulate VSMC tone is probably the vascular endothelium, which is the principal source of PGI_2, EDRF and endothelins (Lüscher, 1990).

1.8. *Contraction of vascular smooth muscle*

The process of VSMC contraction is not as completely understood as that of skeletal muscle and there are many differences between these two types of muscle. In this section, I will discuss the different components involved in VSMC contraction.

1.8.1. *Role of Ca^{2+} in smooth muscle contraction*

One of the earliest intracellular events in VSMC contraction is a transient increase in $[Ca^{2+}{}_i]$. As was the case for platelets (see Section 1.4.), increases in $[Ca^{2+}{}_i]$ can occur either as a result of a Ca^{2+} influx from the extracellular fluid or by an $Ins(1,4,5)P_3$-induced Ca^{2+} release from an intracellular store (reviewed by Bean, 1989). In VSMC the intracellular Ca^{2+} store that is involved in this agonist-induced release of Ca^{2+} is the sarcoplasmic reticulum. Fluorescent indicators have been used successfully to measure basal and stimulated levels of VSMC $[Ca^{2+}{}_i]$

(reviewed by Bean, 1989). Using fura-2 the basal [$Ca^{2+}{}_{i}$] in arterial smooth muscle was estimated to be about 100 nM (Goldman *et al.*, 1989). Also, Ca^{2+} ions are uniformly distributed in smooth muscle cells at rest (Goldman *et al.*, 1989). This resting level of [$Ca^{2+}{}_{i}$] is very similar to that found in platelets (Section 1.4.). Upon stimulation of VSMC with agonists or Ca^{2+} ionophores, the [$Ca^{2+}{}_{i}$] levels can increase to levels exeeding 1 μM in a very short time (Goldman *et al.*, 1989; reviewed by Bean, 1989).

1.8.2. *Ca^{2+}-influx*

Ca^{2+} influx in VSMC can occur by the actions of agonists on receptor-operated Ca^{2+}-channels or via voltage-operated Ca^{2+}-channels upon depolarization. VSMC have different types of receptor-operated Ca^{2+} channels but very little is known about the mechanisms by which VSMC receptor-operated Ca^{2+} channels are regulated (reviewed by Hosey and Lazdunski, 1988). Using patch clamp techniques, ATP has been shown to open a Ca^{2+}-channel in rabbit ear artery (Benham and Tsien, 1987). These ATP-regulated channels were shown to be selective for bivalent cations, and could be opened when the membrane was depolarized. Similarly, norepinephrine was shown to depolarize guinea-pig pulmonary artery (Byrne and Large, 1987). In the latter study, the depolarization was shown to result from an increase in membrane conductance. In a more recent report, Byrne and Large (1988) demonstrated that norepinephrine activated an inward current in VSM from the rabbit portal vein. Also, Rüegg *et al.* (1989) have shown that

norepinephrine induces a depolarization of rat mesenteric artery. Thus, Ca^{2+} channels can be regulated directly by agents that cause VSMC contraction. However, given the substantial increase in Ca^{2+} that occurs as a result of the activation of PLC by some of these agents, the importance of these receptor-mediated effects to contraction caused by these agonists is not clear. Ca^{2+} channels that are directly regulated by G-proteins have also been described (reviewed by Birnbaumer, 1990). The mechanism by which these channels are coupled to the G-proteins, and their importance in VSMC activation has not yet been fully assessed.

The voltage-operated Ca^{2+} channels of VSMC can be subdivided into two different types. One type, the L-type Ca^{2+} channel, is a slowly inactivating Ca^{2+} channel that has a large conductance (approximately 25 pS). These channels can be regulated by dihydropyridines (DHP). The second type of voltage-operated Ca^{2+} channel (the T-type Ca^{2+} channel) is a rapidly inactivating Ca^{2+} channel of low conductance (approximately 9 pS) (reviewed by Hosey and Lazdunski, 1988). As their name implies, the voltage-operated Ca^{2+} channels open upon VSMC depolarization. Depolarizations to > -10 mV cause the opening of L-type Ca^{2+} channels, whereas depolarizations beyond -70 mV will activate T-type channels. Voltage-operated Ca^{2+} channels can be activated by agonists that increase VSMC tone. Thus, receptor-mediated increases in Ca^{2+}- or Na^{+}-permeability could depolarize the membrane and activate the voltage-operated channels.

Behman and Tsien (1988) reported that norepinephrine increased the L-type Ca^{2+} current in rabbit ear artery VSMC. This effect was enhanced by addition of GTP but was not abolished by prior treatment of the cells with propranolol, prazosin or phentolamine. Thus, the receptor involved in this effect of norepinephrine was not a classical α- or β-adrenergic receptor. Also, Rüegg *et al.* (1989) reported that norepinephrine-induced depolarization of rat mesenteric artery could open L-type Ca^{2+} channels. Also, it is perhaps significant that Byrne and Large (1988) have reported a norepinephrine-induced increase in membrane conductance and suggest that this effect may be mediated by Ca^{2+}-activated Cl^- channels, but do not provide any evidence for this conclusion.

1.8.3. *Ca^{2+} release*

A large number of agents that cause VSMC contraction do so by activating VSMC PLC and generating $Ins(1,4,5)P_3$ and DAG (see Section 1.3.3.). Agonist-stimulated PLC activity has been reported in many different VSM types (Smith *et al.*, 1984; Hashimoto *et al.*, 1986; Rapoport, 1986; Lang and Lewis, 1989; Hirata *et al.*, 1990). For example, norepinephrine induced a concentration-dependent increase in the inositol phosphate content of rat aorta (Rapoport, 1986) and both GTP and nonhydrolysable GTP analogues have been shown to stimulate VSM PLC activity (Hirata *et al.*, 1990). The formation of DAG in VSM

following incubation with angiotensin II has also been reported (Griendling *et al.*, 1986). In the last study, the increase in DAG formation correlated with reductions in PIP and PIP_2. Much less is known about the subcellular localization of the PLC involved in signal transduction in VSM. However, Hirata *et al.* (1990) demonstrated a membrane-associated, G-protein-activated PLC activity in cultured bovine aortic smooth muscle cells. This enzyme is probably responsible for the increased $Ins(1,4,5)P_3$ formation in response to most agonists.

$Ins(1,4,5)P_3$ causes a release of Ca^{2+} from intracellular non-mitochondrial stores (reviewed by Berridge, 1987). An $Ins(1,4,5)P_3$ receptor has recently been purified from bovine aortic smooth muscle (Chadwick *et al.*, 1990). This $Ins(1,4,5)P_3$ receptor was shown to contain a 240 kDa polypeptide. Although this receptor is structurally similar to the ryanodine channel of cardiac and skeletal muscle, these two receptors shared only very limited homology (Chadwick *et al.*, 1990). $Ins(1,4,5)P_3$-induced Ca^{2+} release has been reported in skinned VSM (Suematsu *et al.*, 1984) and was shown to be increased by GTP (Saida and van Breemen, 1987; Saida *et al.*, 1988). In skinned smooth muscle, there is also evidence that Ca^{2+} itself can mediate Ca^{2+} release. In addition, this Ca^{2+}-induced Ca^{2+} release can reduce the $Ins(1,4,5)P_3$-induced Ca^{2+} release (reviewed by Bean, 1989). Also, PKC has been suggested to stimulate Ca^{2+} release, since a DAG analogue can mimic the effects of agonists in VSM (Clapp *et al.*, 1987). The time course of release of Ca^{2+} by photoactivation

of caged Ins(1,4,5)P_3 in skinned VSM is consistent with a direct effect by this agent on Ca^{2+} channels (reviewed by Somlyo and Somlyo, 1990). Agonist-induced Ca^{2+} release has also been studied using photoactivable caged compounds. The time course of the release of intracellular Ca^{2+} by caged phenylephrine and caged GTPγS is consistent with their need to activate smooth muscle PLC before they release Ca^{2+} (reviewed by Somlyo and Somlyo, 1990).

1.8.4. *Role of MLC and MLCK in vascular smooth muscle contraction*

A rapid, though transient, increase in [$Ca^{2+}{}_i$] is very important for the contraction of VSM (see Section 1.8.2.). Ca^{2+} does not regulate the formation of tone-generating myosin crossbridges by the same mechanism as in skeletal muscle. Rather, this Ca^{2+}, upon binding to calmodulin, is responsible for the activation of VSM MLCK (Dabrowska *et al.*, 1977). Smooth muscle myosin has 2 heavy chains (200 kDa) and 2 pairs of light chains (20 kDa and 17 kDa) and the MLCK specifically catalyses the phosphorylation of the 20 kDa light chain (reviewed by Kamm and Stull, 1985; Hai and Murphy, 1989). The interaction between myosin heads and actin that occurs following the MLCK-induced phosphorylation of MLC is the basis of VSM contraction and has been extensively studied. Myosin Mg^{2+}ATPase can only be activated by actin when the MLC is phosphorylated (reviewed by Kamm and Stull, 1985; Hai and Murphy, 1989). Thus, by this mechanism of myosin phosphorylation, the number of myosin heads interacting with actin and

the rate at which these crossbridges cycle is greatly increased. Several studies have demonstrated a good correlation between the Ca^{2+}-dependent phosphorylation of myosin and the initial generation of isometric force (reviewed by Kamm and Stull, 1985; Hai and Murphy, 1989). Thus, contraction of the VSM occurs as a result of the force generated by the cycling crossbridges.

Based on this model, relaxation might be expected to result when the $[Ca^{2+}{}_i]$ decreases to a point that could no longer maintain a sufficiently high level of phosphorylated MLC. Dephosphorylation of the MLC by a specific phosphatase would then return the system to the basal state. Such phosphatases have been described in VSM (DiSalvo *et al.*, 1983). However, in most smooth muscle, MLC phosphorylation returns to values indistinguishable from its pre-stimulated level shortly after the establishment of a stable contraction, with little of no effect on the level of tone. $[Ca^{2+}{}_i]$, though decreased, remains somewhat above basal levels. To account for this phenomenon, a Ca^{2+}-dependent, dephosphorylated latch state has been proposed, known as the latchbridge (Dillon *et al.*, 1981; Kamm and Stull, 1985; Hai and Murphy, 1989). This would allow a constant tension to be maintained with little or no need for work to be performed. Also, this latch state is similar to the catch state, proposed to account for the low amounts of energy required by the muscles that keep molluscan shells closed (Rüegg, 1986).

1.8.5. *PKC-mediated phosphorylation and sustained VSM contraction*

Recently, PKC-induced phosphorylation of VSM proteins has been proposed to account for the sustained contraction observed with many contractile agents (reviewed by Rasmussen *et al.*, 1987). The proteins involved in this process could include intermediate filament proteins such as α-desmin, β-desmin, and synemin (Rasmussen *et al.*, 1987) and thin filament regulatory proteins, such as caldesmon (Tanaka *et al.*, 1990) and calponin (Winder and Walsh, 1990). Thus, incubation of bovine carotid artery with histamine or a phorbol ester for one hour results in the establishment of a stable contraction and the phosphorylation of a large number of cellular proteins (Takuwa *et al.*, 1988). The proteins phosphorylated after this one hour incubation are called "late proteins" (Takawa *et al.*, 1988). In contrast, angiotensin II, which induced only a transient increase in smooth muscle DAG and did not promote the PKC-mediated phosphorylation of these late proteins, did not cause a sustained contraction (Takawa *et al.*, 1988). Similar results were obtained in experiments with tracheal smooth muscle (Park and Rasmussen, 1986).

The phosphorylation by PKC of caldesmon (Tanaka *et al.*, 1990) and calponin (Winder and Walsh, 1990) may also have effects on muscle tone. Thus, the actin-binding proteins caldesmon and calponin have been shown to inhibit crossbridge formation in the absence of Ca^{2+} and calmodulin (Adam *et al.*, 1989). Sustained PKC-mediated

phosphorylation of these proteins in the contracted muscle may therefore inhibit their reassociation with actin and assist in the maintenance of a stable contraction.

1.9. *Inhibition of VSM function by cyclic nucleotides*

In a situation analogous to that discussed for platelets (see Section 1.5.), cGMP was initially thought to be involved in smooth mucle contraction and cAMP in relaxation. This conclusion was based on correlations between contractions of VSM and tissue cGMP content (Schultz *et al.*, 1973). However, it is now generally accepted that both cGMP and cAMP are mediators of VSM relaxation, rather than contraction (Lincoln, 1989; Walter, 1989; Murray, 1990).

1.9.1. *cAMP- and cGMP-mediated protein phosphorylation in VSMC*

There is surprisingly little information concerning the phosphorylation of cellular proteins in response to increases in either cyclic nucleotide in VSM. Earlier work utilizing crude particulate fractions of smooth muscle had shown that cGMP could alter the level of phosphorylation of certain membrane proteins (Casnellie *et al.*, 1980). Rapaport *et al.* (1982) was the first to demonstrate that compounds that increase cGMP in intact cells altered the level of phosphorylation of cellular proteins. In this work, SNP and EDRF caused phosphorylation of four proteins, and decreased the level of

phosphorylation of two others. Although it is now clear that one of these proteins was MLC (Draznin *et al.*, 1986), the identity of the others remains unknown. Also, isoproterenol and cAMP analogues increased the level of phosphorylation of many of the same proteins (Rapaport *et al.*, 1982). A 135 kDa membrane protein shown to be a cGMP-PK substrate by Casnellie *et al.* (1980) was also phosphorylated by cAMP-PK (Parks *et al.*, 1987). There has been considerable speculation over the identity of this phosphoprotein. Early work using purified VSMC plasma membrane (Furukawa and Nakamura, 1987) or VSMC microsomal fractions (Rashatwar *et al.*, 1987) had shown that phosphorylation of a 135 kDa protein correlated with an increased Ca^{2+} uptake in both systems. Also, an antibody to the platelet cGMP-PK was shown to inhibit this effect (Popescu *et al.*, 1985). However, the Ca^{2+}ATPase and the cGMP-PK substrate have been shown to have different electrophoretic mobilities (Baltensperger *et al.*, 1988). Moreover, purified Ca^{2+}ATPase is not a substrate for purified VSMC cGMP-PK (Lincoln, 1989). However, it is possible that during the purification of the Ca^{2+}ATPase (Lincoln, 1989) an important regulatory component was lost. The activation of the Ca^{2+}ATPase from skeletal muscle by phosphorylation of protein-bound phosphatidylinositol (Varsanyi *et al.*, 1983) is consistent with this possibility. Vrolix *et al.* (1989) have recently reported a similar effect in VSM.

1.9.2. *Effects of cAMP and cGMP on [$Ca^{2+}{}_i$] in VSMC*

A transient increase in [$Ca^{2+}{}_i$] is central to the contraction of VSM. Increases in [$Ca^{2+}{}_i$] can arise as a consequence of increased Ca^{2+} uptake from the extracellular space (see Section 1.8.2.), or increased Ca^{2+} release from the sarcoplasmic reticulum (Section 1.8.3.). There are many mechanisms by which [$Ca^{2+}{}_i$] is regulated in VSMC and these are similar to those studied in platelets (Section 1.5.2.). The transient increase in [$Ca^{2+}{}_i$] (Morgan and Morgan, 1982) is inhibited by agents that increase intracellular cGMP (Morgan and Morgan, 1984; Hassid, 1986; Rashatwar *et al.*, 1987). Similarly, agents that increase the intracellular level of cAMP also inhibit the increases in [$Ca^{2+}{}_i$] (Kai *et al.*, 1987; Felbel *et al.*, 1988; Abe and Karaki, 1989; DeFeo and Morgan, 1989). Thus, both cyclic nucleotides can inhibit VSMC contraction through effects on Ca^{2+} movements.

1.9.2.1. *Effects of cAMP and cGMP on Ca^{2+} entry into VSMC*

The uptake of Ca^{2+} into VSMC is inhibited by cGMP (Taylor and Meisheri, 1986) and cAMP (Meisheri and van Breemen, 1982). Also, analogues of either cyclic nucleotide inhibit Ca^{2+} currents in cultured VSMC from both rat and rabbit aorta (Ousterhaut and Sperelakis, 1987; Bkaily *et al.*, 1988). The L-type Ca^{2+} channel isolated and purified from cardiac and skeletal muscle is a substrate for cAMP-PK and

this phosphorylation reduces its frequency of opening (Curtis and Catterall, 1985; Flockerzi *et al.*, 1986). Whether the VSM Ca^{2+} channel is a substrate for either of the cyclic-nucleotide activated protein kinases is not known.

1.9.2.2. *Effects of cAMP and cGMP on phosphatidylinositol hydrolysis and the mobilization of Ca^{2+} by Ins(1,4,5)P_3*

Ins(1,4,5)P_3-induced release of Ca^{2+} from the sarcoplasmic reticulum is an important component of many agonist-mediated contractions (Section 1.8.3.). Agents that increase the levels of cGMP have been shown to inhibit the formation of inositol phosphates in VSM (Rapoport, 1986; Chuprun and Rapopórt, 1987; Fijii *et al.*, 1986; Lang and Lewis, 1898; Hirata *et al.*, 1990). In early work, the effects of these agents on the total inositol phosphates formed in response to contractile agents were measured (Rapoport, 1986; Chuprun and Rapoport, 1987). More recently, nitrovasodilators, EDRF and cGMP analogues have been shown to reduce the Ins(1,4,5)P_3 formation in VSMC stimulated with norepinephrine and other contractile agents (Lang and Lewis, 1989; Hirata *et al.*, 1990). Although the molecular basis for the cGMP-mediated inhibition is not clear, an effect on the G-protein that couples the receptors to the PLC is likely. Thus, Hirata *et al.* (1990) showed that cGMP also inhibited the response to non-hydrolysable GTP analogues. These workers also investigated the effects of cAMP on the PLC activity of VSMC and showed that, though

inhibitory, this cyclic nucleotide was much less effective than cGMP.

Cyclic nucleotide-mediated inhibition of the Ins(1,4,5)P_3-induced release of Ca^{2+} from the sarcoplasmic reticulum has been described (Meisheri *et al.*, 1986; Collins *et al.*, 1986). Although the mechanism involved was not elucidated, the cyclic nucleotides were also able to inhibit the caffeine-induced release of Ca^{2+}. The Ins(1,4,5)P_3 receptor purified from brain is a cAMP-PK substrate and phosphorylation of this protein reduced its ability to mediate the release of Ca^{2+} (Supattapone *et al.*, 1988). Recently, the Ins(1,4,5)P_3 receptor isolated from bovine aortic smooth muscle was shown to be structurally and functionally similar to the brain receptor (Marks *et al.*, 1990). Thus, cAMP and perhaps cGMP may be able to reduce Ca^{2+} mobilization by Ins(1,4,5)P_3 receptors in VSMC.

1.9.2.3. *Effects of cAMP and cGMP on Ca^{2+} removal from the cytosol*

[$Ca^{2+}{}_i$] can be reduced by either active transport of Ca^{2+} ions out of the cell or back into the sarcoplasmic reticulum. There are two plasma membrane systems that can participate in Ca^{2+} removal, the Ca^{2+}ATPase and the Na^+/Ca^{2+} exchanger. The relative contributions of these two systems is a matter of contention (Schatzmann, 1989; van Breemen and Saida, 1989).

Agents that increase cGMP have been reported to increase Ca^{2+} efflux from VSMC (Itoh *et al.*, 1983; Collins *et al.*, 1986; Rashatwar *et al.*, 1987; Furukawa *et al.*, 1987, 1988). Although this effect was associated with an increased Ca^{2+}ATPase activity (Rashatwar *et al.*, 1987), and the effect was blocked by an antibody to cGMP-PK (Popescu, 1985), the Ca^{2+}ATPase itself was not phosphorylated (Baltensperger *et al.*, 1988). However, the increased Ca^{2+}ATPase activity could be related to the phosphorylation of phophatidylinositols by a PI kinase (see Section 1.9.1.). Agents that increase cAMP and cAMP analogues have no such effect on Ca^{2+} efflux or on Ca^{2+}ATPase activity (reviewed by Murray, 1990).

There are many reports of cAMP- or cGMP-induced increases in Ca^{2+} uptake by the sarcoplasmic reticulum, or by vesicles prepared from these membranes (Saida and van Breemen, 1984; Hwang and van Breemen, 1987; Twort and van Breemen, 1988; Komori and Bolton, 1989). However, the molecular basis of these effects is not clear. Although phospholamban has been reportedly purified from VSM, and is a substrate in vitro for both cyclic nucleotide-activated kinases (Watras, 1988; Raeymaekers *et al.*, 1988; Huggins *et al.*, 1989), this protein is not phosphorylated as a result of increases in cGMP in intact tissue (Huggins *et al.*, 1989). cAMP-PK and cGMP-PK-mediated increases in Ca^{2+} uptake by sarcoplasmic reticulum vesicles from porcine coronary artery have been reported (Suematsu *et al.*, 1984). The cAMP-mediated event was associated with the phosphorylation of 22 kDa

and 28 kDa proteins. In this system, cGMP-mediated phosphorylation of a 35 kDa protein was also reported (Suematsu *et al.*, 1984).

1.9.2.4. *Effects on the contractile apparatus*

Phosphorylation of MLC by MLCK is central to contraction of VSMC (Section 1.8.4.). The VSM MLCK is phosphorylated at a common site by both cAMP-PK and cGMP-PK (Adelstein *et al.*, 1978; Johnson and Lincoln, 1985; Hathaway *et al.*, 1985; Paglin *et al.*, 1988). Since this phosphorylation reaction has been shown to reduce the affinity of MLCK for Ca^{2+}/calmodulin, it was suggested that it may mediate relaxation of VSMC by reducing the MLC phosphorylation. However, since only phosphorylation of MLCK in the absence of Ca^{2+}/calmodulin affected enzyme function, this effect is of uncertain physiological relevance in relaxation (Miller *et al.*, 1983; Gerthoffer *et al.*, 1984; Sellers and Adelstein, 1987). However, it is established that increases in cAMP in VSM block phosphorylation of MLC (Rapoport, 1982; Silver, 1985) and a role for phosphorylation of MLCK in this effect cannot be ruled out. Thus, cyclic nucleotides have been shown to regulate VSM tone without affecting $[Ca^{2+}{}_i]$ (Nishimura and van Breemen, 1989). This was demonstrated by using permeabilized VSMC containing a buffered $[Ca^{2+}{}_i]$. The authors concluded that the cyclic nucleotides affect function by decreasing the sensitivity of the contractile proteins to $[Ca^{2+}{}_i]$.

1.10. *Adenylyl cyclase system*

Adenylyl cyclase catalyses the conversion of ATP to cAMP. There are probably several forms of adenylyl cyclase, and most have molecular masses of 120-150 kDa (Smigel, 1986). It is now generally accepted that at least three functionally distinct classes of proteins interact in the adenylyl cyclase system. These components are receptors, GTP-binding proteins (G-proteins), and the catalytic unit. Extensive purification and reconstitution studies have demonstrated that all three components are required for the normal activation of the enzyme (Ross and Gilman, 1980; May *et al.*, 1985; Feder *et al.*, 1986).

1.10.1. *Receptors*

Much of the earlier work on receptors coupled to adenylyl cyclase was carried out with adrenergic receptors (reviewed by Lefkowitz and Caron, 1988). Whereas both the β_1 and the β_2-adrenergic receptors are coupled to adenylyl cyclase through G_s, the α_1 and α_2-adrenergic receptors have different mechanisms of action. α_1-adrenergic receptors are coupled to phospholipase C through an as yet unidentified G-protein, and α_2-adrenergic receptors are negatively coupled to adenylyl cyclase through G_i, most probably G_{i2}

(Lefkowitz and Caron, 1988, Freissmuth *et al.*, 1989; Simonds *et al.*, 1989). From detailed analysis of the deduced sequences of these receptors many common features have been noted. First, all the receptors have the potential, based on hydrophobicity calculations, to form 7 membrane-spanning domains (Lefkowitz and Caron, 1988). The homology of the different receptors is greatest in these putative transmembrane sequences (Lefkowitz and Caron, 1988). Based on analysis of point mutations and α/β-adrenergic receptor chimeras, it has been suggested that these transmembrane sequences are important in agonist binding. The third cytoplasmic loop, as well as the cytoplasmic C-terminal segment, appear to be involved in the interaction of the receptor with the G-protein (Lefkowitz and Caron, 1988, Collins, *et al.*, 1989). It is now clear that all receptors coupled to effectors through G-proteins show structural characteristics similar to those of the α- and β-adrenergic receptors.

Several different types of receptors activate or inhibit adenylyl cyclase in platelets and VSMC. These receptors are all coupled to adenylyl cyclase through G_s or G_i, respectively (reviewed by Freissmuth, 1989; Birnbaumer, 1990). Receptors coupled to adenylyl cyclase through G_s include those for PGE_1 and PGI_2 (IP receptors), PGD_2 (DP receptors) and adenosine (A_2 receptors) (reviewed by Williams, 1987; Halushka *et al.*, 1989). Receptors coupled to inhibition of platelet adenylyl cyclase through G_i include those for thrombin, PAF and epinephrine (α_2-adrenergic receptors) (reviewed

by Lefkowitz and Caron, 1988; Siess, 1989). Similarly, adenosine, PGE_1, PGI_2, PGE_2, vasoactive intestinal peptide (VIP) and calcitonin-gene related protein (CGRP) activate VSMC adenylyl cyclase through specific receptors and G_s, whereas α_2-adrenergic agonists inhibit VSMC adenylyl cyclase through G_i (reviewed by Lefkowitz and Caron, 1988; Murray, 1990).

Platelet receptors of particular importance in this thesis are the receptors for PGE_1 and prostacyclin (PGI_2) and the A_2-adenosine receptor. Based on results of binding experiments and protein purification, Dutta-Roy and Sinha (1987) showed that PGE_1 and PGI_2 activate platelet adenylyl cyclase through the same receptor. This PGE_1/PGI_2 receptor is an IP type of receptor (reviewed by Halushka *et al.*, 1989). Also, the PGE_1/PGI_2 receptor, as purified from NCB-20 somatic hybrid cells, has a molecular mass of 83 kDa (Leigh *et al.*, 1989). Ashby, has proposed that platelets have two distinct PGE_1 receptors coupled to adenylyl cyclase; one activates the enzyme (the IP receptor) whereas the other slowly inhibits it (Ashby, 1988; 1989a). Although some thromboxane A_2 antagonists have been shown to have inhibitory effects similar to that of PGE_1 (Ashby, 1989b), there is no established molecular basis for the presense of an inhibitory receptor with these characteristics. There are many purinergic receptors on mammalian tissues and these have very distinct mechanisms of action (reviewed by Williams, 1987). An A_2-adenosine receptor was recently purified from bovine brain and shown to be a 45 kDa protein

(Barrington *et al.*, 1989). Since neither the PGE_1 or the A_2-adenosine receptors have yet been cloned, very little information is available on their structures. However, several reports have demonstrated that the activation of adenylyl cyclase by PGI_2 or adenosine is GTP-dependent (Murray *et al.*, 1989; reviewed in Freissmuth *et al.*, 1989).

1.10.2. *Receptor-G-protein interactions*

The G-proteins involved in the regulation of adenylyl cyclase are membrane-associated heterotrimeric proteins (reviewed by Freissmuth *et al.*, 1989, and Birnbaumer, 1990). One of the subunits, α, binds GTP and has GTPase activity, whereas the β and γ subunits have regulatory roles. When agonists activate adenylyl cyclase in a receptor-dependent manner, the receptors interact with G_s, the G-protein that stimulates adenylyl cyclase. Upon binding of an agonist to its receptor, the rate at which GDP dissociates from $G_s\alpha$ is increased. Although the mechanism of this increased dissociation is not clear, it results in an exchange of bound GDP for GTP (reviewed by Freissmuth *et al.*, 1989; Birnbaumer, 1990). Once the $G_s\alpha$ subunit is occupied by GTP, the ternary complex (agonist-receptor-G-protein) dissociates and the affinity of the receptor for its agonist is reduced. It is generally agreed that the "free" receptor is then available to bind to another molecule of G_s and then to interact with another molecule of agonist. Upon dissociation of the ternary complex,

the α-subunit (containing bound GTP) is free to interact with and activate the catalytic subunit of adenylyl cyclase. Activation of the enzyme is terminated by the intrinsic GTPase activity of $G_s\alpha$, which then reassociates with $\beta\gamma$. So far as is known, the interactions of receptors with other heterotrimeric G-proteins all conform to this same model.

1.10.3. *Interactions of G_s and G_i with the catalytic subunit of adenylyl cyclase*

Very little is known about the mechanism by which the $G_s\alpha$-GTP causes activation of adenylyl cyclase. What is clear, however, is that this subunit activates adenylyl cyclase until the GTP is hydrolysed to GDP. Thus, the GTPase activity of the G-protein is the rate-limiting step in the activation process. Since the rate of GTP hydrolysis by $G_s\alpha$ is slow ($<10\ min^{-1}$), the rate of inactivation is equally slow (reviewed in Levitzki, 1987). However, when the agonist in question mediates a receptor-dependent inhibition of adenylyl cyclase, rather than activation, the G-protein involved, probably G_{i2} (Simonds *et al.*, 1989), and the mechanisms by which it interacts with adenylyl cyclase are different and complex. Although the first step in the process, GDP-GTP exchange, is identical to that involved in enzyme activation, and the $G_i\alpha$-subunit probably dissociates from the heterotrimeric G_i, it may be the $\beta\gamma$ subunits of this complex that exert most of the inhibitory effect by interacting with and

inhibiting activated $G_s\alpha$-subunit (reviewed by Freissmuth *et al.*, 1989). The precise mechanism by which the $\beta\gamma$ from G_i brings about this inhibition is not clear, but one possibility would involve stabilizing of the GTP-bound form of G_s. However, direct effects of the $G_i\alpha$ or the $\beta\gamma$-subunits on adenylyl cyclase activity are also likely (reviewed by Birnbaumer, 1990).

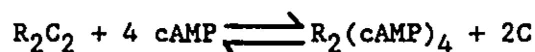
1.10.4. *Catalyst*

Forskolin, a diterpine that has been extensively used to study the effects of cAMP, activates adenylyl cyclase (Seamon and Daly, 1981) by interacting directly with the catalytic unit (Laurenza *et al.*, 1989). Recently, the purified 120 kDa adenylyl cyclase catalyst was cloned from bovine brain and expressed in COS-m6 cells. Transfection of the full length cDNA elevated the adenylyl cyclase activity in these cells (Krupinski *et al.*, 1989). The deduced amino acid sequence predicts a 124 kDa polypeptide with potentially 12 membrane-spanning domains (Krupinski, *et al.* 1989). Two intracellular domains within the protein sequence are highly homologous to each other and to the catalytic domain of guanylyl cyclases (Krupinski, *et al.*, 1989).

1.11. *cAMP-dependent protein kinase (cAMP-PK)*

cAMP-activated protein kinases (cAMP-PK) have been described in almost every cell type studied. cAMP-PK catalyses the transfer of the

gamma-phosphate of ATP to protein Ser or Thr residues. The enzyme is tetrameric with separate regulatory (R) and catalytic (C) subunits, which dissociate in the presence of cAMP (reviewed in Beebe and Corbin, 1986; Edelman *et al.*, 1987).



$$R_2C_2 + 4\ cAMP \rightleftharpoons R_2(cAMP)_4 + 2C$$

The free catalytic subunit catalyses the phosphorylation reaction. It is generally accepted that there are two main types (Type I and Type II) of cAMP-PK based on elution profiles from ion exchange columns and sequence data (reviewed by Beebe and Corbin, 1986; Edelman *et al.*, 1987). The Type I enzyme has a molecular mass of 167 kDa and the Type II kinase a molecular mass of 172 kDa. Both types of cAMP-PK have very similar catalytic subunits. The molecular mass of the catalytic subunit isolated from different sources is 38 kDa. The regulatory subunits have been shown to differ significantly (reviewed by Beebe and Corbin, 1986; Edelman *et al.*, 1987). The molecular masses of the two regulatory subunits, as deduced from sequence data, are 43 kDa (R_I) and 45 kDa (R_{II}). Also, the catalytic and regulatory subunits can be further subdivided, based both on sequence data and tissue distribution (reviewed in McKnight *et al.*, 1988). There are no differences in the catalytic activities of any of the different isozymes of the cAMP-PK (reviewed in McKnight, *et al.*, 1989), although there are differences in the kinetics of subunit dissociation (reviewed by Edelman *et al.*, 1987). In platelets (Salama and Haslam, 1984) and in VSM (Silver *et*

al., 1987), both soluble and particulate cAMP-PK activities have been reported. In platelets, the Type I enzyme is membrane-bound and the Type II enzyme is soluble (Salama and Haslam, 1984), whereas the subcellular distribution of these isozymes in VSM is variable (Edelman *et al.*, 1987).

The cyclic nucleotide binding sites of cAMP-PK are located on the C-termini of the regulatory subunits. There are subtle differences in the affinities of these sites (reviewed by Beebe and Corbin, 1986; Edelman *et al.*, 1987). The site from which cAMP dissociates more slowly is referred to as site I and that for which cAMP dissociates more quickly is referred to as site II. Activation of the kinase by two analogues, each selective for only one site, is synergistic. This is a consequence of the positive cooperativity exhibited by the two sites (reviewed by Beebe and Corbin, 1986; Edelman *et al.*, 1987). Each subunit of both the Type I and Type II enzymes can be phosphorylated by various kinases (reviewed by Beebe and Corbin, 1986; Edelman *et al.*, 1987). Also, R_{II} is autophosphorylated at Ser 95. This autophosphorylation has been shown to inhibit reassociation of R_{II} with C (reviewed by Beebe and Corbin, 1986; Edelman *et al.*, 1987). Although R_I can be phosphorylated at Ser 99 by the cGMP-PK, the physiological relevance of this effect is not clear (reviewed by Edelman *et al.*, 1987).

1.12. *Synthesis and function of cGMP*

1.12.1. *Synthesis of cGMP*

The enzyme responsible for the formation of cGMP is guanylyl cyclase. This enzyme catalyses the metabolic conversion of GTP to cGMP and pyrophosphate. It is clear that up to three types of guanylyl cyclase are present in many mammalian cells (reviewed by Waldman and Murad, 1987). These enzymes can be separated on the basis of their sub-cellular distribution, their kinetic parameters, and the different mechanisms by which they are regulated (reviewed by Waldman and Murad, 1987; Tremblay *et al.*, 1988; Shulz *et al.*, 1989). Although almost all cells contain both soluble and particulate guanylyl cyclase activities, and some a form that is bound to the cytoskeleton, the amount of each type is cell specific. Platelets are unusual in that they contain only the cytosolic guanylyl cyclase activity (Adams and Haslam, 1978). Most types of smooth muscle contain both soluble and particulate guanylyl cyclase activities (reviewed by Waldman and Murad, 1987).

1.12.2. *Soluble Guanylyl Cyclase*

Soluble guanylyl cyclase is a heterodimer with a molecular mass of 150 kDa. The subunits of the dimer have masses of 70 kDa and 82 kDa, respectively. Recently, a substantial amount of progress has been made

in elucidating the structure of the enzyme (Kamisaki *et al.*, 1986; Koesling *et al.*, 1988; Nakane *et al.*, 1988; Nakane *et al.*, 1990). Both the 70 kDa and the 82 kDa subunits of the heterodimer have been purified and cloned (Nakane *et al.*, 1990; Harteneck *et al.*, 1990) and are coded for by different genes. A 200 amino acid domain in the carboxy-terminal region of each subunit was homologous to the putative catalytic domain of the adenylyl cyclase (Krupinski *et al.*, 1989). Although transfection of L-cells with either of the subunits alone did not increase guanylyl cyclase activity, simultaneous transfection with both subunits did so (Nakane *et al.*, 1990; Harteneck *et al.*, 1990). Thus, expression of both subunits was required for guanylyl cyclase activity.

The active form of guanylyl cyclase contains a heme residue. Loss of this prosthetic heme group reduces the basal activity and renders the enzyme unresponsive to nitric oxide (Gerzer *et al.*, 1981; Ignarro *et al.*, 1989). Iron (Fe^{2+}) and copper (Cu^{2+}) are both necessary for enzyme function (Gerzer *et al.*, 1981), and activation of the enzyme by nitric oxide is dependent on the oxidation state of the iron (reviewed by Ignarro, 1989). However, the role of copper in the catalytic activity of this enzyme remains unclear. Activation of the enzyme requires GTP in a bivalent cation-complexed form (reviewed by Waldman and Murad, 1987). Although in vitro many bivalent cations can be used, Mg^{2+} is most probably the physiological ion. However, most experiments are carried out with MnGTP because of

the higher rate of catalysis that can be obtained with Mn^{2+} (reviewed by Waldman and Murad, 1987). In addition, excess free bivalent metal ions are also required for catalysis. Again, Mg^{2+} is thought to serve this function (reviewed by Waldman and Murad, 1987). Activators of soluble guanylyl cyclase are numerous and include nitric oxide and other free radicals, oxidized fatty acids, and protoporphyrin IX. Although there is a large amount of information available concerning activation of soluble guanylyl cyclase by oxidized fatty acids and protoporphyrin IX (reviewed by Waldman and Murad, 1987), the physiological relevance of these effects is unclear.

Activation of soluble guanylyl cyclase by nitric oxide involves a complex series of interactions between the free radical and the iron containing heme-enzyme complex (reviewed by Ignarro, 1989). These interactions, which are still poorly understood, bring about a marked change in the kinetic properties of the enzyme. The largest effect, and that thought to be the basis for the increased catalysis, is an increase in the V_{max} (reviewed in Waldman and Murad, 1987; Ignarro, 1989). While the basal activity follows Michaelis-Menten kinetics, once activated, the enzyme shows a more complex kinetic behaviour (reviewed by Waldman and Murad, 1987). A recent model of the nitric oxide-induced activation of guanylyl cyclase proposes that the interaction of nitric oxide free radical with the iron causes the iron to move out of the plane of the porphyrin ring (reviewed by Ignarro, 1989). Since heme containing displaced iron is structurally similar to protoporphorin IX,

Ignarro (1989) concludes that this displacement is necessary for activation.

There are a large number of compounds that can activate soluble guanylyl cyclase by releasing nitric oxide, including $NaNO_2$, hydroxylamine, nitroglycerin, SNP, isosorbide dinitrate, SIN-1, nitrosamines and amylnitrite (reviewed by Waldman and Murad, 1987). The largest and best characterized group of pharmacological agents that act in this manner are the nitrovasodilators. These compounds have been shown to have significant effects on many cell types, including smooth muscle and platelets (reviewed by Murad, 1986; Lincoln, 1989; Walter, 1989). It is now widely accepted that the nitrovasodilators act by releasing nitric oxide, either spontaneously or enzymatically, and that their effects are the result of activation of soluble guanylyl cyclase by nitric oxide. The physiological counterpart to the nitrovasodilator is endothelium-derived relaxing factor (EDRF) (reviewed by Angus and Cocks, 1989). This factor, initially described by Furchgott and co-workers (Furchgott and Zawadzki, 1980) as a highly labile factor produced and released by endothelial cells, has been the focus of much study. The release of EDRF from endothelial cells can be stimulated by a broad range of agents including acetylcholine, ADP, ATP, adenosine, serotonin, bradykinin, substance P, thrombin and Ca^{2+} ionophore (A23187) (reviewed by Angus and Cocks, 1989). Although the mechanism by which the release of EDRF is coupled to receptor activation is uncertain a rise in [$Ca^{2+}{}_i$] has been shown to be of central importance

(reviewed in Angus and Cocks, 1989). EDRF has been identified as nitric oxide (Palmer *et al.*, 1987) or S-nitrosocysteine (Myers *et al.*, 1990) and is synthesized from L-arginine. EDRF is constantly released at low levels from vascular endothelium, even in the absence of specific stimuli (reviewed by Angus and Cocks, 1989). The enzyme that catalyses the conversion of L-arginine to nitric oxide is called nitric oxide synthase. This enzyme is activated by Ca^{2+}/calmodulin (Bredt and Snyder, 1990) and similar activities have been reported in endothelial cells (Palmer *et al.*, 1988), smooth muscle cells (Woods *et al.*, 1990), platelets (Radomski *et al.*, 1990 a,b), and many other types of cells (reviewed in Angus and Cocks, 1989).

1.12.3. *Particulate guanylyl cyclase*

Inconsistent activation of guanylyl cyclase by nitric oxide led investigators to propose that distinct regulatory mechanisms might control the different isozymes (reviewed by Waldman and Murad, 1987). In the early 1980s, factors that were isolated from medium in which sea urchin eggs had been placed (egg-conditioned medium) were shown to be potent activators of particulate guanylyl cyclase in these cells (Hansbrough and Garbers, 1981; Garbers *et al.*, 1982). Two of these factors were called speract and resact. Shortly after the discovery that speract and resact were small peptides, a 28 amino acid peptide released by the atria of rat and human in response to increases in blood volume, and capable of controlling blood pressure, was shown to

stimulate increases in cGMP in many tissues (Hamet *et al.*, 1984; Waldman *et al.*, 1984). This atrial-derived natriuretic factor (ANF) is now called atrial natriuretic peptide (ANP). More recently, other natriuretic peptides have been isolated from brain and heart (Sudoh *et al.*, 1988, 1990; Furuya *et al.*, 1990). All these peptides are structurally very similar, but they have been shown to act in a species and tissue-specific manner. Three peptides (speract, resact and ANP) were specifically crosslinked to membrane-bound proteins that were shown to co-purify with guanylyl cyclase activity (Paul *et al.*, 1987; Shimomura *et al.*, 1986). Based on the co-purification of the 120 kDa ANP-receptor and particulate guanylyl cyclase, Kuno and colleagues concluded that "both ANF binding and guanylyl cyclase activities reside in the same macromolecular complex" (Kuno *et al.*, 1986). However, ANP also binds to a protein with a molecular mass of 70 kDa. This ANP-receptor has been termed the "clearance receptor" and is thought to control the plasma level of ANP by removing the hormone from the circulation (Fuller *et al.*, 1988).

Over the last 10 years, much progress has been made in the elucidation of the structure and function of the particulate guanylyl cyclase. Purification of the enzyme from sea urchin sperm, as well as from rat lung, showed that both enzymes are glycoproteins with molecular masses of 120-150 kDa (Garbers and Radany, 1981; Kuno *et al.*, 1986). Recently, the membrane-bound guanylyl cyclases from sea urchin spermatozoa, rat brain, and human kidney have been cloned (Thorpe and

Garbers, 1989, Chinkers *et al.*, 1989, Lowe *et al.*, 1989). The deduced amino acid sequences indicate that all these enzymes have highly homologous cytoplasmic regions, but that their extracellular ligand-binding domains are very different. Garbers and colleagues have deduced that there are two cytoplasmic domains (Schulz *et al.*, 1989). One, near the carboxy-terminus, is homologous to the putative catalytic domains of soluble guanylyl and adenylyl cyclases (Schulz *et al.*, 1989). The second, which is more N-terminal, shows homology with the catalytic domain of the receptor family of tyrosine kinases (Schulz *et al.*, 1989). Although protein tyrosine kinase activity has not been demonstrated, deletion of this region inhibited stimulation of guanylyl cyclase activity by ANP but not the basal catalytic activity of the enzyme (Chinkers and Garbers, 1989). Transfection of COS-7 cells with cDNA encoding the ANP-receptor/guanylyl cyclase polypeptide resulted in a large increase in both ANP binding and guanylyl cyclase activity in the transfected cells (Lowe *et al.*, 1989). Recently, based on results obtained with specific antibodies and from radiation inactivation experiments, an inhibitory component of this system has been suggested (Ishido *et al.*, 1989, Ohuchi *et al.*, 1989). The nature of this component, and its relationship to the ANP/GC molecular complex is not clear.

1.13. *cGMP-dependent protein kinase (cGMP-PK)*

The first mammalian cGMP-stimulated protein kinase activity was

isolated from rat cerebellum by Hofmann and Sold (1972). Although this kinase is not as ubiquitous as the cAMP-PK (see Section 1.11.), it has been described in many mammalian tissues. Also, it is present in significant amounts in platelets, smooth muscle and liver (reviewed by Beebe and Corbin, 1986; Edelman *et al.*, 1987; Tremblay *et al.*,1988; Walter, 1989). Although cGMP-PK activity is usually soluble, particulate forms have been described (e.g. platelets and VSM) (reviewed by Walter, 1989). Although more than 80% of the cGMP-PK activity of platelets is associated with a particulate fraction, much less (25%) of the enzyme present in rat and rabbit aortic smooth muscle is particulate (Ives *et al.*, 1980; Walter, 1981). Another cGMP-PK that is membrane-bound is the Type II enzyme found in the porcine intestinal brush border (DeJonge, 1981). Whether the platelet enzyme and the Type II enzyme are related has not been investigated.

The bovine lung soluble cGMP-PK is the most extensively studied isoenzyme and is referred to as Type I. This enzyme is a homodimer with a native molecular mass of 150 kDa (reviewed by Beebe and Corbin, 1986; Waldman and Murad, 1987). The monomer has been sequenced and contains two cGMP-binding sites, as well as a catalytic domain (Takio *et al.*, 1984). cGMP-PKs isolated from other sources are both immunologically and structurally similar to the bovine lung enzyme (reviewed by Edelman *et al.*, 1987). In contrast to activation of cAMP-PK, activation of the cGMP-PK does not involve dissociation of the homodimers (reviewed by Beebe and Corbin, 1987; Edelman *et al.*, 1987).

Recently, two different isozymes of the Type I enzyme have been described in bovine aorta (Wolfe *et al.*, 1989a). These enzymes have been called Type Iα and Type Iβ; the monomer of Iα has a molecular mass of 78 kDa, whereas that of the Iβ isozyme has a molecular mass of 80 kDa. The Iα monomer was indistinguishable from the subunit of the lung enzyme, whereas the 80 kDa monomer of the Iβ enzyme was different (Wolfe *et al.*, 1989a). The kinetics of dissociation of cGMP from the two binding sites of the Iβ enzyme were slower than from the Iα sites (Wolfe *et al.*, 1989a). Also, based on limited sequence analysis, it is clear that the Iα and Iβ monomers have have different N-terminal sequences (Wolfe *et al.*, 1989a).

1.13.1. *cGMP binding sites*

Each monomer contains two cGMP-binding sites (reviewed by Beebe and Corbin, 1986; Edelman *et al.*, 1987). These sites are homologous to the cAMP-binding sites of the cAMP-PK, as well as to other cyclic nucleotide binding sites on other proteins (e.g. the catabolite gene activator protein of E. coli) (Takio *et al.*, 1984). The binding selectivity of these sites for cGMP rather than cAMP has been shown to be due to the presence of a Thr rather than an Ala residue (Weber *et al.*, 1989; Shabb *et al.*, 1990). It has been suggested that hydrogen bonding between the hydroxyl group of this Thr residue and the 2-amino group of cGMP stabilizes the interaction. The Ala residue in

the cAMP binding-site of cAMP-PK would not allow such H-bonding to occur (Shabb *et al.*, 1990). Although the two cyclic nucleotide binding sites present in this protein are selective for cGMP, they do have slightly different affinities for cGMP and its analogues. One site (site I) binds cGMP with higher affinity than site II and is referred to as the slowly exchanging site (reviewed by Beebe and Corbin, 1986). Using cGMP analogues, it was shown that binding of cGMP to these sites demonstrates positive cooperativity (reviewed by Beebe and Corbin; Edelman *et al.*, 1987). Thus, a synergistic activation of this kinase similar to that seen with the cAMP-PK (Section 1.11.) was achieved with analogues of different selectivity.

1.13.2. *Catalytic domain*

Based on sequence homology, the catalytic domain of cGMP-PK is thought to reside at the C-terminal end of the protein (Takio *et al.*, 1984). Consistent with this, proteolyzed forms of the cGMP-PK lacking N-terminal sequence retain catalytic activity and are sometimes constitutively activated (Wolfe *et al.*, 1989b).

Autophosphorylation of the cGMP-PK has been extensively studied (reviewed by Beebe and Corbin, 1986; Edelman *et al.*, 1987). It has been shown that cyclic nucleotides that bind with lower affinity than cGMP (cAMP, cIMP) promote the autophosphorylation of the cGMP-PK (reviewed by Beebe and Corbin, 1986; Edelman *et al.*, 1987). Hofmann

and Flockerzi (1983) demonstrated that both cGMP and cAMP caused autophosphorylation of the kinase. However, significantly (10-fold) higher concentrations of cAMP were required. Also, the extents of phosphorylation caused by cGMP and cAMP are very different. Whereas cGMP-stimulated autophosphorylation caused approximately 1 mol of PO_4 to be incorporated per mol of enzyme, cAMP can stimulate the addition of up to 2-3 mol of PO_4/mol of enzyme (Aitken *et al.*, 1984). The sites phosphorylated are Ser 50, Ser 72, Thr 58 and Thr 84. All of the potential phosphorylation sites of this kinase are located in the N-terminal "hinge region" (reviewed by Edelman *et al.*, 1987). cAMP-mediated autophosphorylation of the cGMP-PK has profound effects on the function of this enzyme causing a 10-fold reduction in the concentration of cAMP required for activation and a 10-fold reduction in the rate of cGMP dissociation from site I (Hofmann and Flockerzi, 1983, Hofmann *et al.*, 1985). The possibility that some cAMP-PK was present during these experiments was not rigorously excluded and it would be of interest to test the effects of cAMP on the very pure preparations of cGMP-PK that are now available (Wolfe *et al.*, 1989a,b).

1.13.3. *Activation*

Much of the early work was based on knowledge acquired from studies of the cAMP-PK (Section 1.11.). Until recently, it was thought that the two identical monomers were positioned in an antiparallel

manner such that one monomer regulated the activity of the other. Although widely accepted (Beebe and Corbin, 1986; Edelman *et al.*, 1987; Tremblay *et al.*, 1988; Walter, 1989), this mechanism was never rigorously tested. Recently, utilizing a proteolytically cleaved form of the bovine aortic Iβ isozyme that retained cGMP-sensitivity, evidence was obtained that the catalytic and inhibitory domains interact primarily within the same subunit, rather than between subunits (Wolfe *et al.*, 1989b). These findings have still to be applied to the function of the unproteolyzed enzyme but imply a new model of cGMP-PK activation (Wolfe *et al.*, 1989b).

1.14. *Cyclic nucleotide phosphodiesterases*

Cyclic nucleotide phosphodiesterases (PDEs) catalyse the hydrolysis of cyclic nucleotides and play very important regulatory roles in cyclic nucleotide-based signal transduction systems (reviewed by Beavo 1988, 1990). Both cyclic 2',5'- and 3',5'-nucleotide PDEs are known to exist in nature but only enzymes of the latter group that preferentially hydrolyse cAMP and/or cGMP will be discussed, since the bulk of the work described in this thesis relates directly to the roles of these two cyclic nucleotides in the control of platelet and VSM function.

Over the last 15 years, there has been a rapid increase in the amount of information available concerning the structures and the

functions of the different cyclic nucleotide PDEs. Also, several mechanisms by which different types of PDEs are regulated have been elucidated (Beavo 1988; 1990). Several different nomenclatures for PDEs have been adopted by different groups (Thompson *et al.*, 1971; Hidaka and Asano, 1976; Weishaar *et al.*, 1986; Beavo *et al.*, 1988). In the earlier systems, PDEs were identified and classified on the basis of their order of elution from ion exchange columns (Thompson *et al.*, 1971; Hidaka and Asano, 1976, Hidaka and Endo, 1984, Weishaar *et al.*, 1986). Since the profiles of PDE activities obtained using these chromatographic procedures tended to be tissue specific, these systems did not allow ready tissue to tissue comparisons of PDE activities, and hence were clearly inadequate. In this thesis, a nomenclature based on very specific criteria will be adopted. These criteria are: 1) the preferred cyclic nucleotide hydrolysed at physiological concentrations; 2) the cofactors required for activity; and 3) the effects of pharmacological inhibitors. This system, taken from two recent reviews (Beavo 1988 and 1990), allows direct comparisons of PDE activities from different tissues, based on their catalytic activities and their regulatory mechanisms, rather than on their affinities for DEAE-cellulose.

Even though the hydrolysis of cyclic nucleotides would normally be regulated in a concerted manner by all the active PDE enzymes present in a particular cell, for the purpose of clarity, the different PDEs will be discussed individually in this Introduction. Special attention

will be directed to the platelet and VSM enzymes and to the physiological and pharmacological effects thought to be mediated by these enzymes.

1.14.1. *Type I: Ca^{2+}/calmodulin-activated phosphodiesterases (Ca^{2+}/calmodulin-PDE)*

As the name implies, this type of PDE is activated by Ca^{2+} and calmodulin. This PDE has been shown to contribute to cyclic nucleotide breakdown in almost every mammalian cell studied. However, in heart and brain the Ca^{2+}/calmodulin-PDE constitutes a large percentage of the PDE activity and has therefore been most extensively studied in these tissues (reviewed by Wang *et al.*, 1990). Both immunological and classical column purification methodologies have suggested the existence of different isozymes of Ca^{2+}/calmodulin-PDE (reviewed by Wang *et al.*, 1990). In one instance, the different isozymes have been shown to arise as a result of alternate splicing of the primary RNA transcript (Charbonneau *et al.*, 1986). Although most of the enzymes of this type are homodimers with native molecular masses of 150 kDa, larger isozymes have been described (reviewed by Wang *et al.*, 1990). *In vitro*, these enzymes can be activated up to 20-fold by Ca^{2+} and calmodulin. Most of the isozymes of this family preferentially hydrolyse cGMP and often have K_m values for cAMP that are at least 50 times higher than those for cGMP (Keravis *et al.*, 1987). However, in some instances (e.g. bovine lung), similar affinities for cGMP and

cAMP have been reported (Sharma *et al.*, 1986).

There have been conflicting reports concerning the presence of this type of cyclic nucleotide PDE in platelets. Hidaka and Asano (1976) and Simpson *et al.* (1988) reported a human platelet cGMP-selective PDE that was potently activated by Ca^{2+} and calmodulin. In marked contrast to these results, Weishaar *et al.* (1986) found that the hydrolysis of cGMP in lysates of human platelets was completely insensitive to the presence of Ca^{2+} and calmodulin. Also, a selective inhibitor of this enzyme, vinpocetine, had no effect thrombin-induced aggregation of human platelets (Hidaka and Endo, 1984).

A Ca^{2+}/calmodulin-PDE activity has been isolated from VSM. This enzyme, sometimes referred to as FII or PDEI, was shown to hydrolyse cGMP preferentially (Weishaar *et al.*, 1986). It has been suggested that activation of this PDE could explain the receptor-mediated Ca^{2+}-dependent decreases in cGMP and cAMP that have observed in some experiments (reviewed by Wang *et al.*, 1990). Also, vinpocetine caused significant reductions in vascular tone in rabbit aorta (Hidaka and Endo, 1984). The effects of Ca^{2+}/calmodulin-PDE inhibitors were markedly enhanced when used on rabbit aorta that had previously been incubated with the contractile agent (Ahn *et al.*, 1989). Thus, these inhibitors were shown to cause significant increases in the levels of smooth muscle cyclic nucleotides in the presence of norepinephrine, but not in the absence of this agent. These authors

concluded that the effects of these compounds would depend on the contractile state of the muscle (i.e. on [$Ca^{2+}{}_i$]) (Ahn *et al.*, 1989).

Activation of the purified enzyme *in vitro* is potently inhibited by calmodulin antagonists. In addition, PDE inhibitors such as IBMX, M&B 22,948 and 1-methyl-3-isobutyl-8-methyl xanthine inhibit both the basal and stimulated activities of the enzyme (reviewed by Wang *et al.*, 1990). The Ca^{2+}/calmodulin-PDE is phosphorylated by both cAMP-PK and Ca^{2+}/calmodulin-dependent protein kinase *in vitro* and although the physiological importance of these phosphorylation reactions are unclear, a working model has been proposed (Wang *et al.*, 1990).

1.14.2. *Type II: cGMP-stimulated cAMP phosphodiesterases (cGS-PDE)*

This enzyme was first described in the soluble fraction of liver (Beavo *et al.*, 1971) but is found in many different types of cells (reviewed by Manganiello *et al.*, 1990). cGS-PDEs isolated from brain and heart are homodimers with native molecular masses of about 200 kDa and a monomeric molecular mass of 105 kDa. Although *in vitro* this enzyme can hydrolyse either cGMP or cAMP with positively cooperative kinetics, at physiological concentrations of these cyclic nucleotides, the enzyme preferentially hydrolyses cAMP (reviewed by Beavo, 1988; Manganiello *et al.*, 1990). In many tissues, there are both soluble and particulate cGS-PDEs. Although these enzymes have

similar kinetics, they are distinct proteins with different primary structures (reviewed by Manganiello *et al.*,1990, Trong *et al.*, 1990).

The stimulation of cAMP hydrolysis by cGMP is mediated by the binding of cGMP to an allosteric site (Wada *et al.*, 1987). This cGMP binding site is homologous to the cGMP binding sites present on many other PDE enzymes (Beavo and Reifsnyder, 1990; Charbonneau, 1990). By binding to this allosteric site, cGMP brings about a reduction in the K_m for cAMP but has no effect on the V_{max} of this enzyme for cAMP (reviewed by Beavo, 1988; Manganiello *et al.*, 1990). Although maximum stimulation of cAMP hydrolysis is obtained with 10 μM cGMP, concentrations of cGMP as low as 80 nM have a significant effect. At high concentrations, cGMP inhibits the hydrolysis of cAMP (Wada *et al.*, 1987; Grant *et al.*, 1990). This effect is presumably due to competition with cAMP for the active centre (reviewed by Manganiello *et al.*, 1990). IBMX and other methyl xanthines have been shown to enhance the binding of cGMP to the non-catalytic cGMP-binding site (Miot *et al.*, 1985; Beavo and Reifsnyder, 1990).

Although a cGS-PDE has been purified from platelets (Grant *et al.*, 1990), no evidence has yet been presented for the presence of the enzyme in smooth muscle. The cGS-PDE purified from human platelets appears to be structurally and kinetically very similar to the heart and brain enzymes (Grant *et al.*, 1990). This platelet PDE is cytosolic and has a monomeric molecular mass of 105 kDa (Grant *et al.*, 1990).

The enzyme, which requires Mg^{2+} for both basal and stimulated catalytic activity, was not affected by addition of Ca^{2+} and calmodulin. Although *in vitro* the platelet enzyme hydrolysed both cGMP and cAMP at roughly equal maximal rates, at physiological concentrations of these cyclic nucleotides, the preferred substrate was cAMP. Although PDE inhibitors such as IBMX or papaverine inhibited the enzyme, 10-fold higher concentrations of these agents were required than for inhibition of the platelet cGMP-inhibited cAMP-PDE (Grant *et al.*, 1990).

This PDE has been shown to play important physiological roles in heart, adrenal cortex and other tissues. In heart, the presence of this enzyme may explain why compounds that increase cGMP reduce cAMP-mediated increases in Ca^{2+} currents (Hartzell and Fischmeister, 1986). In adrenal cortex, cGS-PDE is thought to mediate indirectly the inhibitory effects of ANP on aldosterone production. Thus, in this tissue the enzyme is located only in the region responsible for aldosterone synthesis (zona glomerulosa) (Gallant *et al.*, 1974; MacFarland *et al.*, 1991). A similar effect of ANP on the cAMP content of human fibroblasts has also been reported (Lee *et al.*, 1988).

1.14.3. *Type III: cGMP-inhibited cAMP phosphodiesterases (cGI-PDE)*

Weber and Appleman (1982) first described this activity in rat adipose tissue. Later, Grant and Colman (1984) purified a low K_m cAMP PDE from human platelets that was inhibited by submicromolar

concentrations of cGMP. A similar enzyme was subsequently purified from bovine heart by Harrison *et al.* (1986). The enzyme isolated from heart displayed similar kinetics to the platelet enzyme. Thus, these enzymes hydrolyse cAMP and cGMP with K_m values in the submicromolar range; the V_{max} for cAMP is 10-fold higher than for cGMP. The K_i for inhibition by cGMP is very similar or identical to the K_m for this cyclic nucleotide. This PDE activity is inhibited by the classical PDE-inhibitors, but there is also a group of highly selective inhibitors of this enzyme. These include cilostamide (Hidaka *et al.*, 1979), milrinone (Silver *et al.*, 1988), anagrelide (Gillespie, 1988), siguazodan (Murray *et al.*, 1990), and other inotropic compounds. In fact, a substantial body of evidence suggests that the ability of these compounds to affect cellular function correlates with their ability to inhibit this enzyme (see below and Weishaar *et al.*, 1987).

1.14.3.1. *Platelet cGI-PDE*

A cGI-PDE like activity was first described in platelets by Hidaka and Asano (1976). Later, Grant and Colman (1984) purified this cGI-PDE from human platelets. The enzyme isolated by the latter workers was cytosolic and accounted for over 80% of the total cAMP PDE activity present. This cGI-PDE had an apparent monomeric molecular mass of 61 kDa, as determined by SDS-PAGE, and of 140 kDa, as determined by gel filtration. The K_m values for both cAMP and cGMP were submicromolar and the purified PDE hydrolysed cAMP at a rate of 2.5 μmol/min/mg of

protein.

Using an antibody to the cardiac enzyme, MacPhee *et al.* (1986) demonstrated that the enzyme purified by Grant and Colman (1984) was a proteolysed form of a 110 kDa protein; the intact protein was shown to be highly susceptible to proteolysis (MacPhee *et al.*, 1987). Moreover, cGI-PDE appears to be activated by proteolysis (MacPhee *et al.*, 1987). The platelet cGI-PDE is potently inhibited by the inotropic compounds Ro 15-2041, milrinone and cilostamide, as well as by cGMP. In all cases, IC_{50} values were submicromolar (Ro 15-2041, 0.13 μM; cilostamide, 0.04 μM; milrinone, 0.46 μM; cGMP, 0.13 μM).

Platelet cGI-PDE can be activated by incubation of platelets with high concentrations of PGI_2 or forskolin (Grant *et al.*, 1989; MacPhee *et al.*, 1989). This activation was shown to be due to a cAMP-PK-mediated phosphorylation. The role of this enzyme in the homeostasis of cAMP in platelets has been studied using inhibitors, such as cilostamide and milrinone. Potent inhibition of platelet function by these compounds added alone, or in combination with activators of adenylyl cyclase, has consistently been observed (Hidaka, *et al.*, 1979; Hidaka and Endo, 1984; Machleidt *et al.*, 1985; MacPhee *et al.*, 1986; Murray *et al.*, 1990; Lindgren *et al.*, 1990).

1.14.3.2. *VSM cGI-PDE*

Cilostamide, and other compounds known to inhibit the cGI-PDE relax VSM and potentiate the effects of activators of adenylyl cyclase (Lugnier *et al.*, 1986; Schoeffter *et al.*, 1987; Kauffman *et al.*, 1987; Tanaka *et al.*, 1988; Silver *et al.*, 1988; Harris *et al.*, 1989; Ahn *et al.*, 1989; Silver *et al.*, 1989; Lindgren *et al.*, 1990). Also, cilostamide increases the level of cAMP in VSM (Schoeffter *et al.*, 1987; Lindgren *et al.*, 1990). cGI-PDE has recently been purified from rat and bovine aorta (Rascon *et al.*, 1989). The bovine aortic enzyme had a native molecular mass of 105 kDa, as determined by SDS-PAGE, and cross-reacted with an monoclonal antibody directed against the bovine heart cGI-PDE. Also, the enzyme was kinetically similar to the cGI-PDE enzymes isolated from platelets and heart and was a substrate of cAMP-PK.

1.14.3.3. *Cardiac cGI-PDE*

This cGI-PDE was initially described in heart by Donnelly *et al.* (1978) and later purified from this tissue by Harrison *et al.* (1986). Although the bovine heart cGI-PDE was cytosolic, the subcellular location of this enzyme in cardiac tissue seems to be species-specific (reviewed by Manganiello *et al.*, 1990). The bovine heart isozyme was shown to be immunologically indistinguishable from the platelet enzyme (Harrison *et al.*, 1986). Also, the native molecular

mass and kinetic characteristics of these enzymes were virtually identical. Although both cytosolic and particulate cGI-PDEs can be obtained from heart tissue, it is still not clear whether they represent the same enzyme or distinct proteins (Harrison *et al.*, 1986). Interestingly, cardiotonic compounds have been shown to be much more active in species in which the enzyme is particulate than in those in which it is predominantly soluble (reviewed by Manganiello *et al.*, 1990). In the former species (man, monkey), there is also a very good correlation between the effectiveness of cardiotonic drugs and their ability to inhibit the cGI-PDE (Kauffman *et al.*, 1986; Harrison *et al.* 1986; Ahn *et al.*, 1986). However, there have been no reports that inhibition of this enzyme by cGMP is of physiological or pharmacological importance.

1.14.3.4. *Adipocyte cGI-PDE*

A particulate low K_m cAMP PDE that is potently inhibited by either cGMP or cilostamide has also been purified from rat and bovine adipose tissue (Degerman *et al.*, 1988). In both species, the molecular mass of the enzyme was 110 kDa and the K_m values for cAMP and cGMP were both submicromolar. Although this enzyme was very similar to those previously purified from platelets and heart, the adipocyte cGI-PDE was particulate and hydrolysed cGMP at a relatively faster rate (reviewed by Manganiello *et al.*, 1990). Adipocyte cGI-PDE is activated by lipolytic agents, as well as by insulin (reviewed by

Manganiello *et al.*, 1990). Although it is clear that the lipolytic agents (e.g. glucagon) activate the PDE via a cAMP-PK-dependent mechanism, the reaction that causes activation by insulin is less clear (reviewed by Manganiello *et al.*, 1990). cGI-PDE is synergistically activated when the tissue is simultaneously incubated with lipolytic agents and insulin. Direct inhibition of this enzyme by cGMP in intact cells has not been reported.

1.14.3.5. *"Dense-vesicle" PDE*

Liver contains a large number of distinct PDEs (reviewed by Houslay and Kilgour, 1990). To date, three particulate and five soluble enzymes have been reported. One of the particulate enzymes, the "dense-vesicle" PDE (Heyworth *et al.*, 1983) is a low K_m cAMP-PDE that is potently inhibited by cGMP (Pyne *et al.*, 1987). This enzyme has a native molecular mass of 112 kDa, and a monomeric molecular mass of 60 kDa (Pyne *et al.*, 1987). Antibodies to both the platelet and heart enzymes recognize this liver enzyme (Pyne *et al.*, 1987). Although this enzyme is also activated by insulin, very little is known about the mechanism(s) by which this activation occurs (reviewed by Houslay and Kilgour, 1990).

1.14.4. *Type IV: Ro 20-1724 inhibited cAMP-phosphodiesterases (Rol-PDE)*

This enzyme, a low K_m cAMP PDE, was first purified from dog kidney by Thompson *et al.* (1979). Since this initial description, PDEs with similar kinetics and sensitivity to inhibitors have been described in brain (Davis, 1984; Nemoz *et al.*, 1989), liver (Marchmont *et al.*, 1981), heart (Nemoz *et al.*, 1989; Weishaar *et al.*, 1987), lung (Moore and Schroedter, 1982) and monocytes (Livi *et al.*, 1990). The enzyme from brain and a very similar enzyme from Drosophila (*dnc*$^+$) (Chen *et al.*, 1986), have both been extensively studied. The *dnc* gene, and hence this low K_m PDE is missing in a Drosophila mutant *dnc*$^-$. Loss of the Drosophila enzyme has been related to learning impairment in this species (Chen *et al.*, 1986). Interest in the brain enzyme has been fueled by the fact that the prototypical inhibitor of this enzyme (Ro 20-1724) is a structural analogue of the antidepressant drug, rolipram (Horowski and Sastre-Y-Hernandez, 1984). The enzyme is absent from platelets (Lindgren *et al.*, 1990) but is present in VSM in which it contributes significantly to the hydrolysis of cAMP (Schoeffter *et al.*, 1987). Thus, Ro 20-1724 relaxes VSM and potentiates the effects of activators of adenylyl cyclase (Lindgren *et al.*, 1990).

Rol-PDEs have been extensively studied (reviewed in Conti *et al.*, 1990). The enzyme has a low K_m for cAMP (< 1 μM) and a

relatively high K_m for cGMP (> 50 μM). At low μM concentrations, cGMP does not affect the hydrolysis of cAMP by this enzyme. The V_{max} for cAMP hydrolysis calculated for these enzymes is high, in most preparations around 1 μmol/min/mg of protein. The actual molecular masses of the native enzymes, as determined by conventional biochemical methods, are still a matter of contention. Values between 45 and 135 kDa have been obtained, whereas estimates of subunit molecular masses range from 45 to 74 kDa (reviewed by Conti *et al.*, 1990). Recently, an RoI-PDE from human monocytes was cloned; the cDNA encoded a 686 amino acid (approximately 77 kDa) protein (Livi *et al.*, 1990). This enzyme was homologous to isozymes cloned from rat brain, as well as to the Drosophila PDE (Livi *et al.*, 1990). In liver, RoI-PDE activity has been ascribed to a plasma membrane PDE that seems to be regulated by both insulin and glucagon and is distinct from the "dense vesicle PDE" (reviewed by Houslay and Kilgour, 1990).

1.14.5. *Type V: cGMP-specific phosphodiesterases (cGMP-specific PDE)*

1.14.5.1. *Lung and platelet cGMP-specific PDE*

Although these enzymes will hydrolyse cAMP, rates of cGMP hydrolysis can exceed those for cAMP by as much as 50-fold. Originally described in lung and platelets (Hamet and Coquil, 1978), this enzyme has now been reported in a large number of cells (reviewed in Francis

et al., 1990). The lung and platelet enzymes are both homodimers with native and monomeric molecular masses of 177 and 93 kDa, respectively. Both enzymes have very similar kinetic properties (reviewed in Francis *et al.*, 1990). These enzymes also have a high-affinity non-catalytic cGMP-binding site, similar to that described for the cGS-PDE (Beavo and Reifsnyder, 1990). Binding of cGMP to the high-affinity non-catalytic site(s) is stimulated by IBMX (Hamet and Coquil, 1978). The precise mechanism by which methyl xanthines exert this effect is not known. However, it may be that this interaction reflects a mechanism by which catalytic and allosteric domains can communicate (Hamet and Coquil, 1978; Tremblay *et al.*, 1988). The cGMP-specific PDE can be phosphorylated by both the cAMP-PK and cGMP-PK, but phosphorylation is only possible when cGMP is present (reviewed by Francis *et al.*, 1990). Recently, Thomas *et al.* (1990) reported that both cyclic nucleotides induce phosphorylation of a common site.

1.14.5.2. *The smooth muscle enzyme*

cGMP-specific PDE has not been extensively studied in VSM or purified to homogeneity from this tissue. Moreover, in a recent paper, Weishaar *et al.* (1990) reported that the main peak of cGMP-hydrolysing activity in rabbit aortic smooth muscle was actually composed of three distinct enzymes. One of these was a cGMP-specific PDE. This enzyme was not stimulated by Ca^{2+}/calmodulin. The K_m for cGMP was 0.6 μM, whereas the K_m for cAMP was >20 μM. Also,

the enzyme was potently inhibited by the PDE inhibitor M&B 22,948 (zaprinast). M&B 22,948 also causes marked potentiations of the effects of nitrovasodilators (Kukovetz *et al.*, 1979; Martin *et al.*, 1986). Moreover, in aorta, pulmonary artery and coronary artery, M&B 22,948 potentiated the relaxant effects of ANP (Weishaar *et al.*, 1990).

1.14.5.3. *Rod and cone cGMP-specific PDEs*

Rod and cone cGMP PDEs are also members of the cGMP-specific PDE family (reviewed by Gillespie, 1990). However, these enzymes are structurally different from the lung and platelet enzymes (reviewed in Gillespie, 1990). Both the rod and cone cGMP-specific PDE are composed of 3-4 subunits, most of which have been well-characterized. The bovine rod PDE is a tetramer with an $\alpha\beta\gamma_2$ structure (Deterre *et al.*, 1988). The α and β subunits are 88 kDa and 84 kDa proteins, respectively, and analysis of their sequences indicates that they are highly homologous (Tetuja *et al.*, 1990). The 11 kDa γ-subunits are inhibitory, and dissociate when the enzyme is activated (Tuteja *et al.*, 1990). Both the rod and cone cGMP PDEs have high-affinity, non-catalytic cGMP-binding sites (Beavo and Reifsnyder, 1990).

1.14.6. *Structure/function relationships between PDEs of different families*

Detailed analysis of the sequences of many different PDEs has shown that most are chimeric proteins in that they contain multiple functional domains in distinct parts of their overall structure (Beavo, 1988; Beavo and Reifsnyder, 1990; Charbonneau, 1990). Also, analysis of their sequences indicates that many of these regions are highly conserved. One such highly-conserved region is a C-terminal stretch of 250-270 residues (Charbonneau, 1990). Based on sequence homology, this region is thought to represent the catalytic domain (Charbonneau, 1990, Beavo and Reifsnyder, 1990). Similarly, analysis of the sequences of those PDEs that are under allosteric control by cGMP has indicated that there is an N-terminal cGMP-binding region that is also conserved. More information will become available when additional PDEs have been sequenced, but it is already clear that although different PDEs have distinct functions, they are members of a group of structurally related enzymes (Beavo and Reifsnyder, 1990, Charbonneau, 1990).

1.15. Thesis objectives

As described above, the cellular levels of cAMP and cGMP are determined by specific cyclases and PDEs. In most cells, cAMP and cGMP have been assumed to be components of independent parallel second messenger systems. My objectives were to investigate whether there are interactions between cAMP and cGMP in the regulation of platelet and VSMC function.

Chapter 2. *Materials and Methods*

2.1. *Materials*

2.1.1. *Animals*

Male New Zealand White rabbits were obtained from Maple Lane Farms (Clifford, Ont., Canada), and male Wistar-Kyoto (WKY) rats were supplied by Harlan Sprague Dawley (Indianapolis, IN, U.S.A.).

2.1.2. *Radiochemicals and scintillants*

[2,8-^{3}H]Adenine (34-38 Ci/mmol) and [8-^{3}H]guanosine (95 Ci/mmol) were from ICN (Irvine, CA) and [8-^{3}H]guanine (16 Ci/mmol) was from Amersham (Oakville, Ont. Canada). cAMP and cGMP ^{125}I-radioimmunoassay kits, [U-^{14}C]ATP (538 mCi/mmol), [U-^{14}C]GTP (440 mCi/mmol), [8-^{14}C]cAMP (50 mCi/mmol), [2,8-^{3}H]cAMP (35 Ci/mmol) and [^{3}H]hypoxanthine (26 Ci/mmol) were obtained from Du Pont Canada (Mississauga, Ont., Canada). [8-^{14}C]cGMP (52 mCi/mmol), [side-chain-2-^{14}C]5-HT and ACS aqueous counting scintillant were supplied by Amersham (Oakville, Ont., Canada). HP/b scintillant was from Beckman Instruments (Toronto, Ont., Canada).

2.1.3. *Fine chemicals and pharmacological agents*

SNP, (-)-isoproterenol-(+)-bitartrate, phenylephrine, carbachol, protein standard solution, all nucleotides, PGE$_1$, HEPES, PIPES, bovine

serum albumin Fraction V (BSA), rabbit hemoglobin, cyclic-3',5'-nucleotide PDE were all from Sigma (St. Louis, MO, U.S.A.). Digitonin was obtained from Calbiochem-Behring (La Jolla, CA, U.S.A.).

2.1.4. *Chromatographic supplies*

Charcoal (Darco G-60) was from Fisher Scientific (Toronto, Ont. Canada) and Celite 535 from Johns Mansville (New York, NY, U.S.A.). Neutral alumina (WN-3) was obtained from Sigma (St. Louis, MO, U.S.A.) and Dowex-50 resin (AG 50W-X8, 100-200 mesh, H^+ form) from Bio-Rad Laboratories (Mississauga, Ont., Canada). Cellulose MN 300 HR powder, cellulose MN 300 HR prefabricated plates and MN 300 PEI/UV_{254} prefabricated sheets were purchased from Brinkman Instruments (Toronto, Ont., Canada).

2.1.5. *Tissue culture materials and other supplies*

Dulbecco's modified Eagle's medium (DMEM), Hanks balanced salt solution (HBSS), fetal calf serum (FCS), antibiotic-antimycotic solution (lyophilized mixture containing penicillin, streptomycin and amphotericin B) (AAS) and all tissue culture plates, dishes and flasks were purchased from GIBCO Canada Inc. (Burlington, Ont., Canada). Polypropylene microtubes with pestles were from Mandel Scientific (Guelph, Ont., Canada).

2.1.6. *Pharmacological agents supplied as gifts*

SIN-1 was from Casella-Riedel (Frankfurt am Main, Germany), M&B 22,948 from May & Baker Ltd. (Dagenham, U.K.) and Ro 20-1724 from F. Hoffman La Roche (Nutley, NJ, U.S.A.). Cilostamide was generously provided by Dr. H. Hidaka of Nagoya University (Nagoya, Japan) and DDA by Dr. R. A. Johnson of Stonybrook University (Stonybrook, NY, U.S.A.). PAF (Ro 148161, synthetic 1-O-octadecyl-2-O- acetyl-*sn*-glycero-3-phosphocholine) was provided by Dr. H. R. Baumgartner of F. Hoffmann La Roche and Co. (Basel, Switzerland). PAF was disolved in ethanol and then diluted to a concentration of 0.1 mM with 0.154 M NaCl containing 3.5 mg of crystallized BSA/ml and 5 mM HEPES, pH 7.4, before use.

2.2. *Methods*

2.2.1. *Preparation of washed rabbit platelets*

Blood was obtained from pentobarbitol anaesthetized male New Zealand White rabbits (2-3 kg) by cannulation of the carotid artery. The blood was collected into syringes containing 0.175 vol. of acid-citrate- dextrose (ACD) anticoagulant (Aster and Jandl, 1964). Platelet-rich plasma (PRP) was prepared by centrifugation of the blood at 160 g_{av} (280 g_{max}) for 15 min at room temperature. Platelets were isolated by centrifugation of the PRP at 2000 g_{av} (3420 g_{max})

for 15 min and resuspended in modified Tyrode's solution containing 5 mM PIPES (adjusted to pH 6.5 with NaOH), 0.35% BSA, 30-60 μg of apyrase/ml and 0.26 mM EGTA (Ardlie *et al.*, 1970). Platelets were resuspended in 10 ml of the modified Tyrode's solution/rabbit. The suspension was centrifuged at 1400 g_{av} (2500 g_{max}) for 10 min and the platelets resuspended in the same Tyrode's solution, but without EGTA, at a platelet concentration of 2.5 x 10^9 platelets/ml. This suspension was used for incubation of platelets with radiolabelled compounds.

2.2.2. *Preparation of washed rat platelets*

WKY rats (225-350 g) were anaesthetized with diethyl ether. The blood was collected into 0.175 vol. of ACD by cardiac puncture using a 23 gauge needle. Preparation of PRP and the subsequent isolation of platelets from the PRP were as described for rabbit blood (Section 2.2.1.).

2.2.3. *Labelling of washed platelets*

Rabbit and rat platelets were isolated by differential centrifugation and washed at room temperature (Section 2.2.1.). The platelets were first incubated at room temperature with 2 μM [^{3}H]guanine (7.4 Ci/mmol, rabbit platelets) or [^{3}H]guanosine (5 Ci/mmol, rat platelets) in order to label the platelet metabolic GTP

pool. Following uptake of about 70% of the [^{3}H]guanine (45-60 min), 4 μM [^{3}H]adenine (4.0 Ci/mmol) was added to the suspension in order to label the metabolic ATP pool. The suspension was incubated for a further 45-60 min, after which approximately 70% of the [^{3}H]adenine had been taken up. It was necessary to add [^{3}H]adenine after [^{3}H]guanine because at these concentrations the former inhibited the uptake of the latter (but not vice versa, Fig. 2.1). In experiments in which platelet cGMP and cAMP were also determined by radioimmunoassay, platelet suspension was incubated with unlabelled 2 μM guanine for 60 min and with 4 μM adenine for a further 60 min, to ensure that the platelets used were as similar as possible to those incubated with the ^{3}H-labelled bases. When required to permit subsequent measurements of platelet degranulation, 1 μM [^{14}C]5-HT was added at the same time as [^{3}H]adenine to label the platelet dense granule contents. Uptake of [^{14}C]5-HT amounted to about 75%. After incubation with either labelled or unlabelled guanine and adenine, the platelets were again isolated by centrifugation and resuspended at 4.2-4.8 x 10^8 platelets/ml in Tyrode's solution (with Ca^{2+}) containing 3-6 μg of apyrase/ml and 5.0 mM HEPES, pH 7.35. This medium was warmed to 37°C before resuspension of the platelets. The actual platelet concentration in the final resuspension solution was chosen to give a platelet concentration of 4 x 10^8 platelets/ml when samples were incubated with drugs.

2.2.4. *Platelet incubation conditions*

Samples of platelets were incubated at 37°C with additions giving a final volume of 0.5 - 1.2 ml. Additions were dissolved in 0.154 M NaCl where possible, or in DMSO (M&B 22,948, cilostamide and Ro 20-1724). The final concentration of DMSO (maximum of 0.2% v/v) was the same in all samples in each experiment in which it was used. When cyclic [^{3}H]nucleotides were determined, incubations were terminated by mixing part or all of each sample with sufficient ice-cold trichloroacetic acid to give a final concentration of 5% (w/v). [^{14}C]cGMP and [^{14}C]cAMP (approximately 700 d.p.m. of each) were then added to each sample to permit measurement of the recovery of each cyclic nucleotide. In some rabbit platelet experiments, 0.5 ml of each incubation mixture was also utilized for measurement of platelet aggregation or the release of [^{14}C]5-HT (Section 2.2.5.).

2.2.5. *Measurement of platelet aggregation and degranulation*

These were studied at 37°C in a Payton Aggregation Module (Payton Associates, Scarborough, Ont., Canada). Samples of platelet incubation mixture (0.5 ml containing 2 x 10^{8} platelets) were stirred in siliconized aggregometer tubes with 10 μl of buffer with or without 10 nM PAF. The extent of aggregation was measured as the decrease in absorbance after 30 s or 60 s. When the release of granule [^{14}C]5-HT was determined, aggregation and degranulation were

terminated by addition of 200 μl of ice-cold 0.154 M NaCl containing 1.5% formaldehyde and 5 mM EDTA. The samples were centrifuged at 12,000 x g for 2 min and 0.5 ml of the supernatant from each sample was transferred to a liquid scintillation vial containing 8.0 ml of HP/b scintillant. This mixture was counted by liquid scintillation to determine the $[^{14}C]$5-HT in the supernatant. The $[^{14}C]$5-HT released by PAF was expressed as a percentage of the total $[^{14}C]$5-HT in the platelets.

2.2.6. *Subculture and labelling of VSMC*

The cultured VSMC were derived from explants of 4 week old WKY rats and supplied by Dr. R.M.K.W. Lee of the Department of Anaesthesia. The cells were grown in 24 (1 cm) well culture dishes. The seeding density was variable, but always allowed confluent cultures to be obtained within 2-3 days. At confluency, each well contained approximately 5×10^5 cells (about 100 μg of protein). Cells were grown in DMEM supplemented with 10% FCS and 1% AAS on the day of subculture. Once confluent, the medium was removed from each well and the cells were covered with 0.5 ml of fresh medium containing $[^{3}H]$hypoxanthine at a concentration of 2 μM and a specific activity of 10 Ci/mmol. Incubation was continued for 16-18 h at 37°C in an incubator with a controlled atmosphere of 95% air and 5% CO_2. Following the incubation, the medium was removed and the cells were washed 3 times with HBSS at 37°C. The cells were then covered with

0.45 ml of fresh HBSS and returned to the incubator before the assay.

2.2.7. *VSMC incubation conditions*

Each well of cultured VSMC was incubated at 37°C with additions to give a final volume of 0.5 ml. Additions were dissolved in either 0.154 M NaCl or DMSO (Ro 20-1724, cilostamide), when necessary. Incubations were terminated by addition of 0.5 ml of ice-cold 10% (w/v) trichloroacetic acid. [^{14}C]cAMP and [^{14}C]cGMP were added to each well and the contents were then transferred to a polypropylene tube.

2.2.8. *Determination of [^{3}H]cGMP and [^{3}H]cAMP in acidified cell extracts*

Acidified samples containing recovery markers were mixed, allowed to stand overnight at 4°C and then centrifuged at 12,000 x g for 2 min. The labelled cyclic nucleotides were isolated from the supernatants by chromatography, first on alumina and then on Dowex 50 resin, using a modification of the method of Jakobs *et al.* (1976). The supernatants were applied to columns containing 1.5 g of alumina that had been washed with 12 ml of 5% (w/v) trichloroacetic acid. The columns were then washed with a further 12 ml of 5% trichloroacetic acid, 12 ml of water and 2 ml of 0.2 M ammonium formate that had been adjusted to pH 6.0 with formic acid. The labelled cyclic nucleotides were then eluted with a further 3 ml of the same ammonium formate

solution. These eluates were acidified with 50 μl of 3 M HCl and applied to columns containing 5 ml (packed vol.) of Dowex 50 resin (Bio-Rad AG 50W-X8) that had been equilibrated with 0.05 M HCl. These columns were then washed with 6 ml of 0.05 M HCl and cGMP was eluted with a further 8 ml of 0.05 M HCl. After washing the columns with 2 x 4 ml of water, cAMP was finally eluted in a further 2 x 4 ml of water. The eluates containing cGMP were neutralized to pH 6 by addition of 50 μl of a 1.6 M (pH 6) HEPES solution and 50 μl of 8 M NaOH. Both the cGMP eluates and those containing cAMP were lyophilized in scintillation vials and counted for ^{3}H and ^{14}C. The labelled cAMP samples were counted in 0.5 ml of water and 8.0 ml of HP/b scintillant, whereas the labelled cGMP samples were counted in a mixture of 0.5 ml of water and 8.0 ml of ACS scintillant, because of their high salt content. Following correction for background, quenching, ^{14}C crossover into the ^{3}H channel and recovery of ^{14}C-labelled cyclic nucleotides (about 50%), the ^{3}H-labelled cyclic nucleotides were usually expressed as percentages of the corresponding ^{3}H-labelled nucleoside triphosphates present in control cells (see Section 2.2.10. and 2.2.11.).

2.2.9. *Determination of the radiochemical purity of cyclic [^{3}H]nucleotides isolated from labelled platelets and VSMC by the dual column procedure*

Platelets were washed and labelled as described in Sections 2.2.1.

and 2.2.3. Samples of suspension containing 2×10^8 labelled platelets were incubated for 0.5 min at 37°C with PGE_1, SNP or both PGE_1 and SNP simultaneously. Similarly, VSMC, labelled as described in Section 2.2.6, were incubated with isoproterenol alone or in combination with either a nitrovasodilator or a cAMP-PDE inhibitor. Incubations were terminated by addition of trichloroacetic acid at a final concentration of 5% (w/v). Known amounts of $[^{14}C]$cAMP and of $[^{14}C]$cGMP (approximately 600 d.p.m. of each) were then added to the acidified extracts. Labelled cGMP and cAMP were then isolated and separated by sequential chromatography on alumina and Dowex 50 resin as previously described (Section 2.2.8). Four identical incubation mixtures were counted for ^3H and ^{14}C and a further 16 samples were processed as described below. The cyclic nucleotide eluates were pooled, dried by rotary evaporation and redissolved in 200 μl of a 2 mM solution of the appropriate cyclic nucleotide. Duplicate 20 μl samples of the latter were further purified by t.l.c. on cellulose using the first dimension solvent described under Section 2.2.10. The cyclic nucleotide spots were eluted with H_2O and counted for ^3H and ^{14}C. A decrease in the ratio of $^3H/^{14}C$ after t.l.c. was taken to indicate the presence of impurities. In order to assess further the purity of the platelet cyclic $[^3H]$nucleotides eluted from the columns, another fraction (90 μl) of the 200 μl sample was incubated with bovine heart cyclic 3',5'-nucleotide PDE. This sample was added to a polypropylene tube containing 10 μl of a concentrated PDE assay mixture, which consisted of 10 mM TES (pH 7.5), 4 mM $MgSO_4$, 0.25 mM EDTA and 0.2 units of PDE/ml. The mixture was incubated for 60 min at

30^oC, after which the reaction was terminated by boiling. Following centrifugation, 20 μl of the reaction mixture was analyzed by t.l.c. on cellulose and the 5'-nucleotide was eluted and counted. The purity of the cyclic nucleotides could be determined from a comparison of the ratio of $^3H/^{14}C$ in the product after PDE treatment relative to that in the starting material.

2.2.10. *Measurement of [^{3}H]GTP and [^{3}H]ATP in labelled platelets*

Samples of control platelet suspension were mixed with 0.2 vol. of 30% (w/v) trichloroacetic acid. [^{14}C]ATP (about 30,000 d.p.m.) was added to one sample and an equal amount of [^{14}C]GTP to another. Both samples were centrifuged at 12,000 x g for 2 min. Trichloroacetic acid was extracted from the supernatants with two times 3 vol. of water-saturated diethyl ether. A sample of each aqueous extract (20 μl) was mixed with 0.1 μmole of unlabelled ATP and GTP, which were then separated by t.l.c. in two dimensions on MN 300 HR cellulose plates. The first dimension solvent was n-butanol:acetone:acetic acid:14.8 M NH_3:H_2O (90:30:20:1:60 by vol.) and the second dimension solvent, isobutyric acid:1 M NH_3:0.1 M EDTA (125:75:2 by vol.). GTP and ATP were eluted from the cellulose with water and counted for ^3H and ^{14}C in HP/b scintillant. Values for platelet [^{3}H]ATP and [^{3}H]GTP were corrected in the same way as the ^{3}H-labelled cyclic nucleotides (Section 2.2.8).

In some experiments the specific radioactivities of the [^{3}H]GTP and [^{3}H]ATP in the platelet metabolic pool were determined in cytosol released by digitonin (Akkerman *et al.*, 1980). This method of solubilization allowed for the selective release of cytosolic components. Very little of the dense granular contents of the platelets were released when low concentrations of the detergent were used (Table 2.1). Washed platelet suspension (10 ml; 4.2 x 10^{9} platelets) was cooled to C^{o}C. The suspension was then mixed with 5 mM EDTA (adjusted to pH 8.0 with NaOH) and 28 μM digitonin added in 50 μl of 0.154 M KCl and incubated for 10 min at 0 ^{o}C. Following removal of the permeabilized platelets by centrifugation (10 min, 160 g_{av}) the supernatant was extracted with 5% (w/v) trichloroacetic acid, recentrifuged (10 min, 2000 g_{av}) and applied to a column containing a mixture of 40 mg of charcoal and 80 mg of celite (in a cold room). After washing the column with water, the nucleotides were eluted with 30 ml of solvent containing 19.2 ml of ethanol, 0.3 ml of 1 M NH_4OH and water to volume. The eluate was dried by rotary evaporation, the nucleotides were redissolved in 50 μl of H_2O and GTP and ATP were isolated by t.l.c. in two dimensions using the method described above. After elution from the cellulose, samples of GTP and ATP were counted for ^{3}H and the amounts present were determined spectrophotometrically, so permitting calculation of their specific radioactivities and of the mass amounts of [^{3}H]cGMP and [^{3}H]cAMP formed from them. The e_M for GTP is 13.7 at 253 nm, whereas that of ATP is 15.4 at 259 nm.

2.2.11. *Isolation of [^{3}H]ATP and [^{3}H]GTP from cultured VSMC*

Control wells of cultured VSMC were extracted by addition of ice-cold trichloroacetic acid (5% (w/v) final). Following addition of about 30,000 d.p.m. of [^{14}C]ATP and of [^{14}C]GTP to the samples, they were transferred to polypropylene tubes which were then centrifuged for 2 min at 12,000 x g. The acidified supernatant was removed and the trichloroacetic acid extracted twice with 3 volumes of water-saturated diethyl ether. Samples of the extracts (50 μl) were counted for ^{3}H in order to determine the total ^{3}H in the extract. Also, other samples (50 μl) were mixed with 0.1 μmole of ATP, GTP, ITP and XTP, which were then separated by two-dimensional t.l.c. on PEI-cellulose sheets, by a modification of the method described by Randerath and Randerath (1964). The first dimension solvent consisted of 1.6 M LiCl and the second, a 1:1 mixture of 2.0 M LiCl and 2 M formic acid. The R_f values determined for the compounds of interest are noted in Table 2.2. A representative two-dimensional separation of the compounds listed in Table 2.2 is shown in Fig. 2.2. The LiCl deposited on the sheets during the first dimension run was removed by a 7 min extraction of the sheets with anhydrous methanol. After separation, both the ATP and the GTP were eluted from the cellulose with 0.3 ml of 0.85 M ammonium formate. Following dilution of the NH_4COOH with an equal volume of water, 0.5 ml of the mixture was counted for both ^{3}H and ^{14}C. The ^{3}H-values were corrected for the recovery of the ^{14}C-labelled compounds and the amount of [^{3}H]ATP and [^{3}H]GTP in

each sample calculated. The range of [^{3}H]ATP and [^{3}H]GTP values found in replicate VSMC cultures was about 5%.

In experiments in which the specific radioactivities of the nucleoside triphosphates were determined, the acidified supernatants from 12 wells (6 x 10^6 cells, 1.2 mg of protein) which had been extracted with trichloroacetic acid were combined and processed through a charcoal column, as previously described for digitonin-permeabilized platelets. The eluate from the column was dried by rotary evaporation, redissolved in 50 μl of H_2O, and the ATP and GTP were isolated by t.l.c. in two-dimensions using the LiCl system. After elution from the PEI-cellulose, the eluates were counted for ^{3}H and the amounts of ATP and GTP determined spectrophotometrically (Section 2.2.10.). The specific radioactivities of ATP and GTP permitted calculation of the mass amounts of the cyclic nucleotides formed from them, after drug treatment of the cells.

2.2.12. *Purification, separation and concentration of platelet and cultured VSMC cyclic nucleotides for radioimmunoassay*

cGMP and cAMP were separated, purified and concentrated by a dual column procedure. Following addition of 3000 d.p.m. of [^{3}H]cAMP and an equal amount of [^{3}H]cGMP to acidified cell extracts, these were applied to columns containing 1 ml of Dowex 50 resin that had been equilibrated with 0.05 M HCl. cGMP was eluted with a further 3 ml of

0.05 M HCl. After washing the columns with 1 ml of H_2O, cAMP was eluted with a further 3 ml of water. Following addition of 50 μl of 3 M HCl to the cAMP eluates, both the cAMP and cGMP eluates were applied to columns containing alumina (0.5 g) that had been washed with 4 ml of 0.2 M sodium acetate (pH 6.2) and 6 ml of 0.05 M HCl. After further washing of these columns with 2 ml of 0.05 M HCl, 4 ml of H_2O and 0.75 ml 0.2 M sodium acetate, the cyclic nucleotides were eluted with a final 1 ml of 0.2 M sodium acetate. The eluates were frozen at -50°C until assayed

2.2.13. *Determination of platelet and vascular smooth muscle cAMP and cGMP by radioimmunoassay*

Cyclic nucleotide values were determined by radioimmunoassay by the method of Harper and Brooker (1975). In this method, the amounts of cAMP and cGMP, in the experimental samples, were determined by interpolation from a standard curve. Standard curves for cAMP were constructed using ^{125}I-labelled 2'-O-succinyl cAMP tyrosine methyl ester ([^{125}I]TME-ScAMP) and different concentrations of 2'-O-acetylated cAMP. Similarly, standard curves for cGMP were constructed using [^{125}I]TME-ScGMP and 2'-O-acetylated cGMP. Radiolabelled succinylated derivatives of the two cyclic nucleotides are the preferred antigens in these assays since the antibodies are raised against 2'-O-succinylated cyclic nucleotide protein conjugates. Although succinylated derivatives would compete more effectively with

the labelled antigens in these systems, technical difficulties make 2'-O-acetylated derivatives preferable (Harper and Brooker, 1975). Representative standard curves for cAMP (Fig. 2.3) and cGMP (Fig. 2.4) are shown. Both the standards and the experimental samples were processed in parallel, thus allowing for direct comparisons of the values obtained in the assays. Samples containing no cells (i.e. trichloroacetic acid acidified buffer blanks) were processed through columns in parallel with the assay samples to correct for effects of impurities in the buffer, trichloroacetic acid or column materials that might affect the binding of the antibody to the labelled cyclic nucleotide derivatives. Similarly, buffer samples to which either 2 or 20 pmol of cAMP and cGMP had been added, were also processed to check that values obtained from the standard curves were reliable. Frozen column eluates were thawed at room temperature and 0.5 ml of each was counted by liquid scintillation in order to estimate the recoveries of the cyclic nucleotides. Of the remaining 0.5 ml, 0.2 ml was acetylated by addition of 2 x 5 μl of an acetylating reagent consisting of a 1:2 mixture of acetic anhydride and triethylamine. Similarly, equal volumes of either cAMP or cGMP standards (4-2000 fmol/200 μl) were also acetylated. Fresh triethylamine was periodically prepared by distillation. After acetylation, 50 μl of each standard and 10, 20 or 50 μl of each experimental sample were added to numbered polypropylene tubes. In those tubes receiving only 10 or 20 μl, the difference in volume was made up with 0.2 M sodium acetate containing acetylating reagent at a concentration of 50 μl/ml. Following

addition of 50 μl of the ^{125}I-labelled succinylated cyclic nucleotide derivative (about 50,000 d.p.m. for cGMP and 25,000 d.p.m. for cAMP), the polypropylene tubes were transferred to an ice bath. Once the tubes had cooled, 50 μl of the antibody solution was added and the contents were mixed using a vortex mixer and incubated in a cold room at 4°C. After 16-18 h (i.e. overnight), 250 μl of an ice-cold precipitating solution was added to the cAMP samples. The precipitating solution consisted of an unidentified mixture provided by the manufacturer to increase the size of the antibody pellet. Similarly, 500 μl of ice-cold 0.2 M sodium acetate was added to the cGMP samples. After the samples had been vortexed and centrifuged for 4 min at 12,000 x g, the supernatant was aspirated and the dried pellet counted for ^{125}I in a gamma-counter for 8 min. The standard curves used in the radioimmunoassays were obtained by plotting the radioimmunoassay data in a logit/log form. The terms for y and x were;

$$y \text{ value} = \text{logit} = \ln[(B/B_o)/(1-B/B_o)];$$

$$x \text{ value} = \log \text{cNMP (fmol)}$$

where B_o and B represent the binding in the absence and the presence, respectively, of a known amount of acetylated cyclic nucleotide. The y-i·.cercept and the slope (m) were then determined by linear regression analysis of the linear portion of this curve. Using these constants and the logit values calculated from the displacement of the [^{125}I]TME-S-cyclic nucleotide in each sample it was possible to

calculate the amount of acetylated cyclic nucleotide present in each of the three or four experimental samples processed. Triplicate dilutions (i.e. 10, 20, or 50 μl) of each experimental sample were assayed to ensure that values which fell within the linear portion of the standard curve were obtained for each experimental sample. Multiple steps were involved in the calculations, and only those logit values which fell on the linear portion of the curve were used. First, the values for the trichloroacetic acid/buffer blanks for the corresponding volume (i.e. 10, 20 or 50 μl) were subtracted. Second, these values were corrected for the actual volume used in the assay thus allowing each sample to be expressed as fmol/ml. Third, samples were corrected for any dilution of the original sample that might have been necessary before the radioimmunoassay. Fourth, the samples were corrected for recovery of cyclic [^{3}H]nucleotide, which in most cases was about 50%. Fifth, the values were corrected for the number of platelets or amounts of VSM protein used in each experimental sample. Lastly, a value for each experimental sample was obtained by taking an average of the values obtained from the triplicate dilutions, and a grand mean for the treatment condition was obtained by averaging the values determined for each experimental condition.

2.2.14. *Preparation of rat aortic rings*

Male WKY rats (225-350 g) were killed by cervical dislocation. The thoracic aorta was rapidly removed and placed in a physiological

salt solution (PSS), containing 1.16 mM $MgSO_4$, 2.5 mM $CaCl_2$, 21.9 mM $NaHCO_3$, 1.16 mM NaH_2PO_4, 4.6 mM KCl, 115.5 mM NaCl and 11.1 mM dextrose, that had been equilibrated with 95% O_2 - 5% CO_2. The aorta was cleaned of adherent fat and connective tissue and cut into 4.0 mm transverse rings. The endothelium was removed by gently rubbing the luminal surfaces with a wooden stick (Furchgott and Zawadzki, 1980). Rings were mounted in 10 ml jacketed organ baths using stainless steel wire and bathed at 37°C in PSS, which was gassed with 95% O_2 - 5% CO_2. Tension development was measured isometrically using a Grass FT03 transducer and a Grass Model 7D polygraph. All experiments were carried out with an initial tension of 2 g, which, in preliminary experiments (Fig. 2.5), was found to be the optimal pre-load tension (Section 2.2.15). Rings were allowed to equilibrate for 2 h, during which they were repeatedly washed with fresh PSS. During this period, the rings were also contracted 3 to 4 times with 30 mM KCl (until a maximal contraction to this concentration of KCl was achieved). Removal of the endothelium was then verified by the inability of 1 μM carbachol to induce relaxation of rings submaximally contracted with 100 nM phenylephrine.

2.2.15. *Determination of the optimal pre-load tension*

De-endothelialized rings of the thoracic aorta of WKY rats were prepared and equilibrated as described in Section 2.2.14. The ability of the rings to contract in response to 30 mM KCl at different

pre-load values was measured. Rings were first challenged with 30 mM KCl at a pre-load tension of approximately 250 mg. Once the contraction had reached a plateau, the KCl was washed out and the rings were allowed to re-equilibrate for 30 min. Following re-equilibration, the pre-load tension was increased and the contraction in response to 30 mM KCl was again recorded. Using this protocol, the ability of the rings to contract in response to 30 mM KCl was measured until a reduction in the maximum contraction induced by this concentration of KCl was noted. Results from one such experiment are shown in Fig. 2.5.

2.2.16. *Studies on the relaxation of aortic smooth muscle by isoproterenol*

The concentration-response curves for the relaxant effects of isoproterenol were determined by cumulative additions of the compound to submaximally contracted rat aortic smooth muscle. In all experiments, contraction was induced by phenylephrine; the concentration used (100 nM) caused on average 80% of the maximal contraction, which was seen with 1 μM. Each addition of isoproterenol was allowed to have its full effect before further additions were made (2 to 3 min). The relaxation caused by each addition was recorded and calculated as a percentage of the maximum possible relaxation (i.e. of the full increase in tension caused by phenylephrine). After 4 washes with PSS, the aortic rings were allowed to equilibrate for 90 min in fresh PSS before they were used again. With this time interval, successive

concentration-response curves for isoproterenol were identical. The effects of nitrovasodilators (SNP or SIN-1) or cAMP PDE inhibitors (cilostamide or Ro 20-1724) on the ability of isoproterenol to relax precontracted aortic smooth muscle were determined as follows. Each compound studied was added to the contracted rings at a specific concentration that caused less than 30% relaxation on all occasions. When the ring tension reached a new plateau (5-15 min), isoproterenol was added cumulatively and the total relaxation caused by each concentration was determined as a percentage of the initial increase in tension caused by phenylephrine. To reduce errors due to the inherent variability of responses of aortic smooth muscle to vasodilators, concentration-response curves for isoproterenol in the absence and presence of each compound were in each experiment constructed from paired records obtained from the same aortic rings.

2.2.17. *Studies on inhibition of the contraction of aortic smooth muscle*

The effects of nitrovasodilators or of cAMP PDE inhibitors on the ability of isoproterenol to inhibit the contractions of rat aortic smooth muscle induced by phenylephrine were studied as follows. Aortic rings were incubated for 30 s with vasodilator compounds, either singly or in combination. Phenylephrine (100 nM) was then added and the tension developed after 2 min was measured. Following each contraction, the PSS was changed 4 times and the rings were allowed to equilibrate in

fresh PSS for 30 min. This time interval permitted the generation of identical control contractions and virtually identical responses to isoproterenol throughout the experiment, provided low concentrations of isoproterenol were studied first. Using this protocol, it was possible to test up to 15 different combinations of compounds on each aortic ring and to obtain concentration-response curves for the inhibition of contraction by isoproterenol in the presence and absence of single concentrations of other compounds. The latter were used at selected concentrations that caused less than a 30% inhibition of contraction. Inhibitions of contraction were calculated as percentages of the control contractions for each aortic ring; 3 or 4 rings received identical treatments in each experiment.

2.2.18. *Analysis of concentration-response curves*

Concentration-response curves were constructed for the relaxation or inhibition of the contraction of rat aortic smooth muscle by isoproterenol in the absence and presence of nitrovasodilators or inhibitors of cAMP PDE. Curves were fitted to the experimental results using a computer program (SigmaPlot 4.02A, Jandel Scientific, Corte Madera, CA, U.S.A.) and the following relationship,

$$E = \frac{E_{max} \cdot x^n}{x^n + I^n} + C$$

where E is the effect, E_{max} the maximum effect, x the concentration of

isoproterenol, I the IC_{50} of isoproterenol, n the Hill slope and C the effect observed in the absence of isoproterenol. This computer program also gave values for the parameters, E_{max}, I, n and C, that minimized the sum of the squares of the differences between the dependent variable (E) in the above relationship and the experimental observations. In addition, a theoretical additive concentration-response curve for isoproterenol in the presence of the other compound tested was constructed as described by Pöch and Holzmann (1980). A leftward shift of the experimental concentration-response curve relative to the theoretical additive curve implies synergism with isoproterenol.

Significant changes in the IC_{50} values of isoproterenol caused by the presence of a nitrovasodilator or an inhibitor of cAMP PDE were evaluated by two-sided paired *t* tests after logarithmic transformation of the results. Supra-additive effects of single concentrations of isoproterenol and other compounds were detected by comparison of the sums of the individual effects with the combined responses, using a repeated measures analysis of the variance of the results from individual aortic rings in at least 3 separate experiments.

2.2.19. *Cyclic nucleotide measurements in rat aortic smooth muscle*

De-endothelialized rat aortic rings were equilibrated in PSS, as described for measurement of the inhibition of contraction, except that the rings were not under tension. Rings were then incubated for 2.5 min

at 37°C in 1.0 ml of PSS containing appropriate concentrations of SNP and/or isoproterenol. Each incubation contained two rings. Aortic rings from different parts of the thoracic aorta were distributed identically in replicates of each incubation condition to prevent any effect attributable to possible differences in their responsiveness to additions. After incubation, the rings were rapidly transferred to polypropylene microtubes precooled in liquid nitrogen. The frozen tissue was immediately pulverized with a precooled polypropylene pestle and then homogenized in 1 ml of 5% (w/v) trichloroacetic acid, following which 6,000 d.p.m. of [^{3}H]cGMP and of [^{3}H]cAMP were added to monitor the recoveries of the two cyclic nucleotides. cGMP and cAMP were then separated and purified by a dual column procedure and finally acetylated and quantitated by radioimmunoassay (Section 2.2.13.). Trichloroacetic acid-precipitated protein was dissolved in 1 M NaOH and assayed as described in Section 2.2.20.

2.2.20. *Protein assays*

In experiments with cultured VSMC, incubations were terminated by the addition of ice-cold trichloroacetic acid (final concentration 5% (w/v)). The precipitated protein was removed from the wells and transferred to polypropylene tubes. When rat aortic rings were used, they were frozen in polypropylene tubes pre-cooled with liquid N_2, pulverized and homogenized in ice-cold 5% (w/v) trichloroacetic acid. In both instances, the tubes were placed on ice for at least 1 h,

following addition of trichloroacetic acid. The acidified supernatants were removed and the protein pellets frozen until the protein assay could be carried out (1-3 days).

Protein was determined by the method of Lowry *et al.* (1951). Protein pellets were disolved in 200 μl of 1 M NaOH. Samples (10, 20 or 50 μl) and the standards were brought to a final volume of 100 μl with 1 M NaOH, before addition of 1.0 ml of a solution containing 2% Na_2CO_3/0.1 M NaOH, 1% sodium potassium tartrate and 0.5% $CuSO_4.5H_2O$ (100:1:1 by vol.). These mixtures were incubated for 15 min at room temperature. Following addition of phenol reagent (100 μl, 2 M) to each tube, the samples were rapidly mixed and incubated for 30 min at 37°C. Absorbance was recorded at 500 nm and, following subtraction of a reagent blank, a standard curve was constructed. In all cases the protein standard solution from Sigma was used. This solution contained 5% human albumin (w/v) and 3% human globulin (w/v). With the spectrophotometer used (Beckman DU-64), subtraction of the reagent blank and calculation of the results from the standard curve were performed automatically. Using this method, it was estimated that one well of cultured VSMC contained approximately 100 μg of protein, whereas one 4 mm rat aortic ring contained about 500 μg of protein.

2.2.21. *Statistics*

In individual experiments, incubations were performed in triplicate or quadruplicate, as indicated, and the results described were observed in at least two separate experiments. Values are given as means ± S.E.M. from either a single experiment or from multiple experiments (see text). All equations are from Knapp, R. G., Basic Statistics for Nurses, 1978, Witney Medical Publications.

$$\text{mean} = Y_m = \sum Y_i/n,$$
$$\text{S.E.M.} = (\sum (Y_i - Y_m)^2/n-1)^{1/2}/n^{1/2}$$

where $\sum Y_i$ is the sum of all the Y values and n the number of measurements.

The significance of changes within experiments was evaluated by two-tailed unpaired *t* tests.

$$t = (Y1_m - Y2_m)/s_p(1/n_1+1/n_2)^{1/2}$$

where $Y1_m$ = mean of set 1, $Y2_m$ = mean of set 2, n_1 and n_2 = number of measurements in sets 1 and 2 ,respectively. s_p is the standard deviation for the two sets and is defined as,

$$s_p = (s_1{}^2(n_1-1) + s_2{}^2(n_2-1)/(n_1+n_2-2))^{1/2},$$

with s_1 and s_2 representing the standard deviation of set 1 and set 2, respectively.

When results from multiple experiments were compared, the significance of changes were in this case evaluated by two-tailed paired *t* tests,

$$t = d_m/(s_d/n^{1/2}); \quad d_m = \sum d_i/n$$

where d_i, is the difference between a pair of values and d_m is the mean difference.

Repeated measures analysis of variance (MANOVA) was used to determine the significance of supra-additive effects of compounds on VSM relaxation and on inhibition of VSM contraction (e.g. Sections 2.2.16 and 2.2.17). This statistical method allows a comparison between groups of numbers in which the degree of variability within each of the groups is such that it invalidates the use of a paired t-test. The analysis used a repeated-measures ANOVA[1] with different experiments as a random between subject factor and two within-subject factors [(ring) and (additive effect vs observed effect)]. The comparison of interest (additive effect vs observed effect) was tested against the two way interaction within each experiment, and F values (with 1 and 2 degrees of freedom) were calculated.

[1] In the actual analysis, a General Linear Model was used because of the imbalance in the design (i.e. some conditions had 3 sets of aortic rings, whereas others had 4).

Table 2.1. Digitonin permeabilization of washed rabbit platelets

Washed labelled rabbit platelets were cooled to $0^{\circ}C$ and treated with the indicated digitonin concentration for 10 min. The amounts of ^{3}H-labelled nucleotides and $[^{14}C]$5-HT released by this procedure were determined by counting the supernatant for ^{3}H and ^{14}C. Values are means ± S.E.M. from 3 determinations in the same experiment.

Concentration of digitonin (mM)	Amount of ^{14}C released (%)	Amount of ^{3}H released (%)
0	2.5	1.6
0.024	10.0	60.0
0.026	9.0	65.7
0.028	13.3	77.2
0.035	18.8	90.0
0.070	65.5	95.5

Table 2.2. R_f values of nucleotides in the two-dimensional t.l.c. method used to separate ATP and GTP from acidified extracts of cultured vascular smooth muscle cells. Values are from Randerath and Randerath (1964).

Compound	1st dimension (1.6 M LiCl)	2nd dimension (2 M LiCl/2 M HCOOH)
ITP	0.413	0.158
XTP	0.180	0.120
ATP	0.340	0.367
GTP	0.287	0.234
IPD	0.640	0.506
XDP	0.360	0.418
ADP	0.560	0.665
GDP	0.466	0.538
IMP	0.753	0.759
XMP	0.467	0.696
AMP	0.640	0.778
GMP	0.593	0.696

Fig. 2.1. Labelling of washed rabbit platelets with [^{3}H]guanine and [^{3}H]adenine

Washed rabbit platelets (2.5 x 10^9/ml) were incubated with radiolabelled adenine (4 μM) in the absence (▨) or presence (■) of 2 μM unlabelled guanine (A), or conversely, with radiolabelled guanine (2 μM) in absence (▨) or presence (■) of 4 μM unlabelled adenine (B). After 60 min at 37°C, the percentages of the labelled bases taken up by these platelets were determined. Values are means ± S.E.M. from 3 identical samples in the same experiment. The effect of adenine on [^{3}H]guanine uptake was significant ($p < 0.05$).

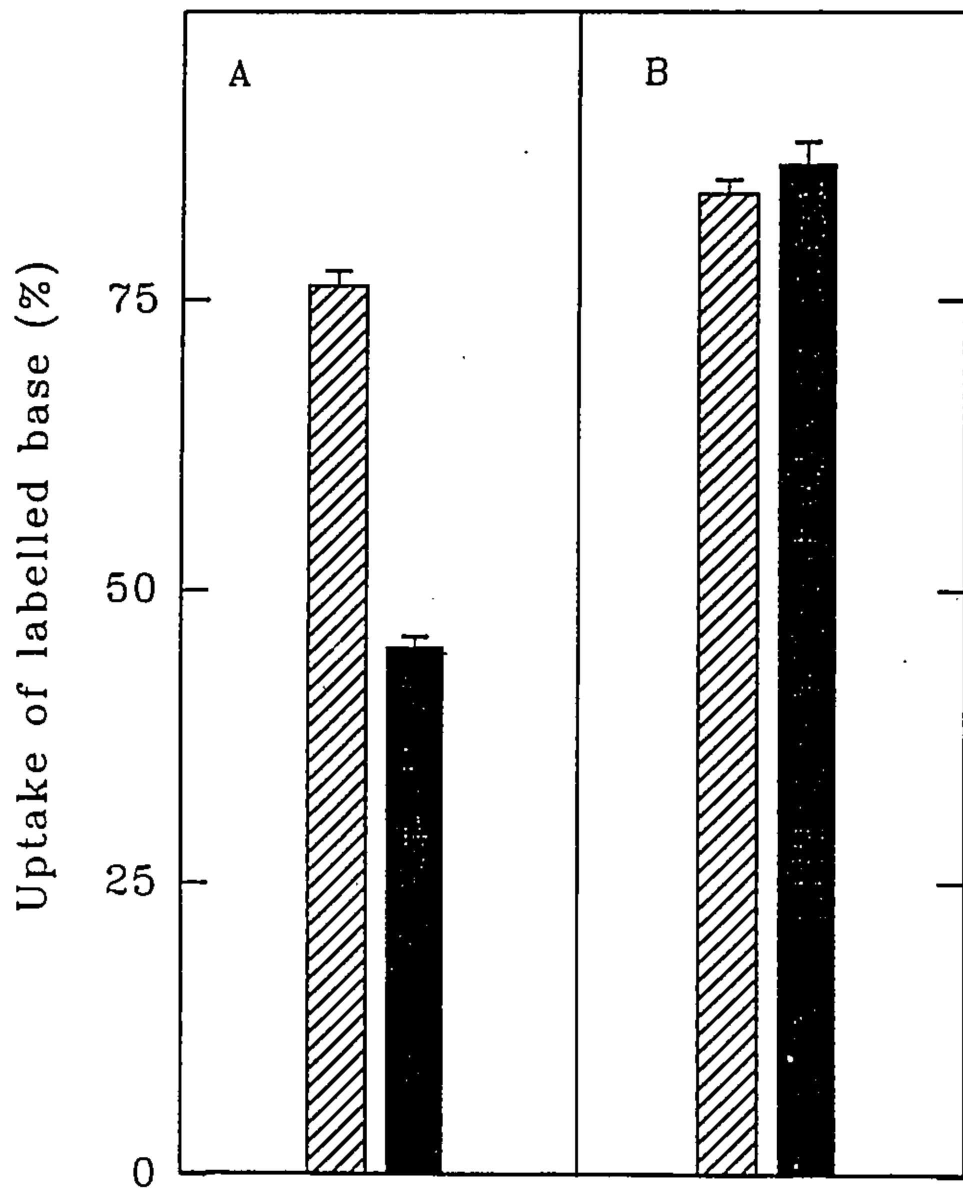

A
B
Uptake of labelled base (%)
75
50
25
0

Fig. 2.2. Representative two-dimensional t.l.c. separation of nucleotides potentially labelled after incubation of cultured VSMC with [^{3}H]hypoxanthine

Equal amounts of each compound (0.1 μmole) were added to the origin of the t.l.c. sheet (O). The first dimension solvent was 1.6 M LiCl and the second was a mixture of 2 M LiCl and 2 M formic acid (1:1). The solvents were allowed to run to within 2 cm of the top of the sheet in each dimension.

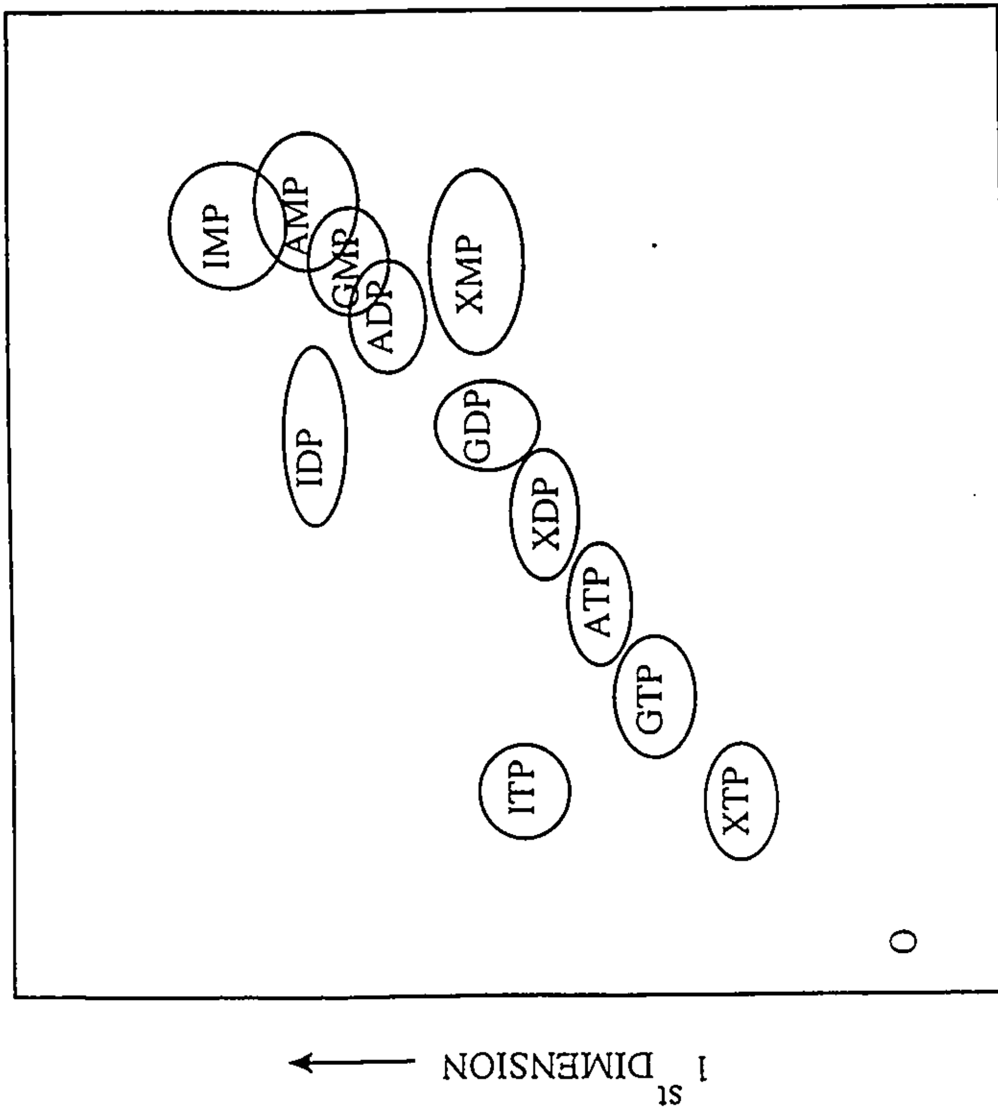
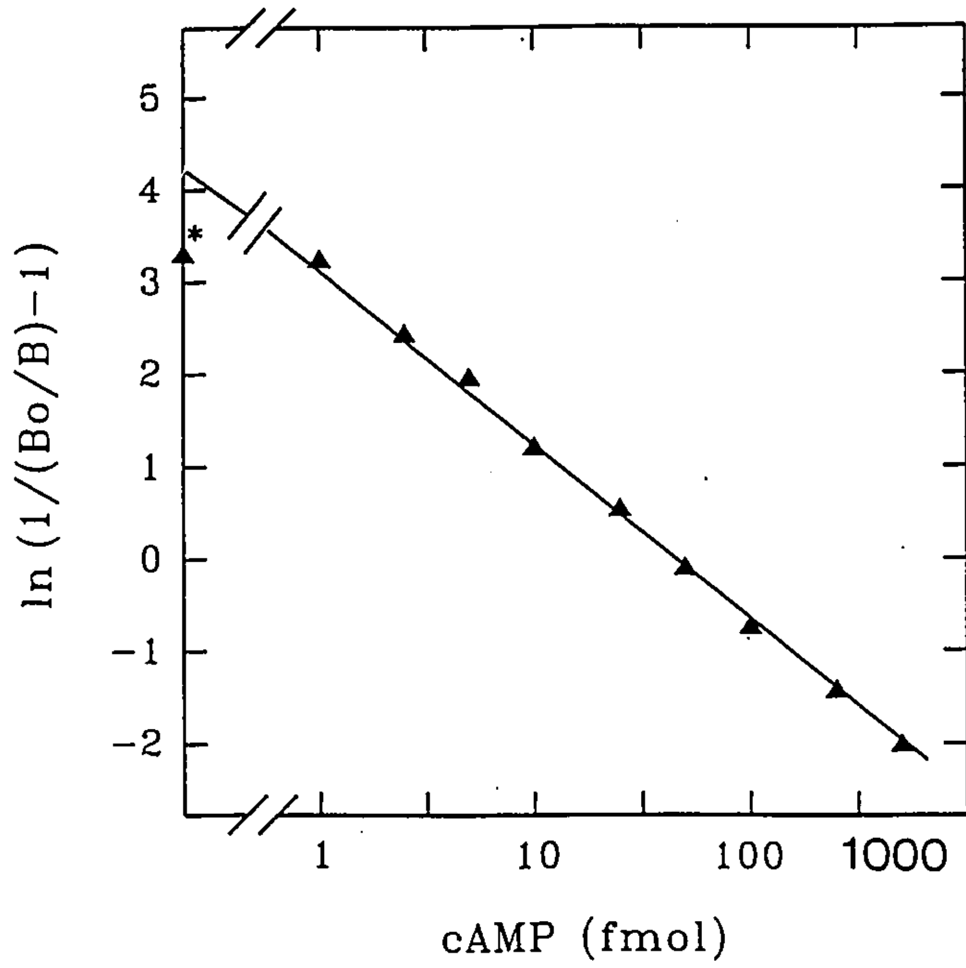

IMP
AMP
GMP
ADP
XMP
IDP
GDP
XDP
ATP
GTP
ITP
XTP
0
1st DIMENSION
2nd DIMENSION

Fig. 2.3. Representative standard curve for the radioimmunoassay of cAMP (Section 2.2.13.)

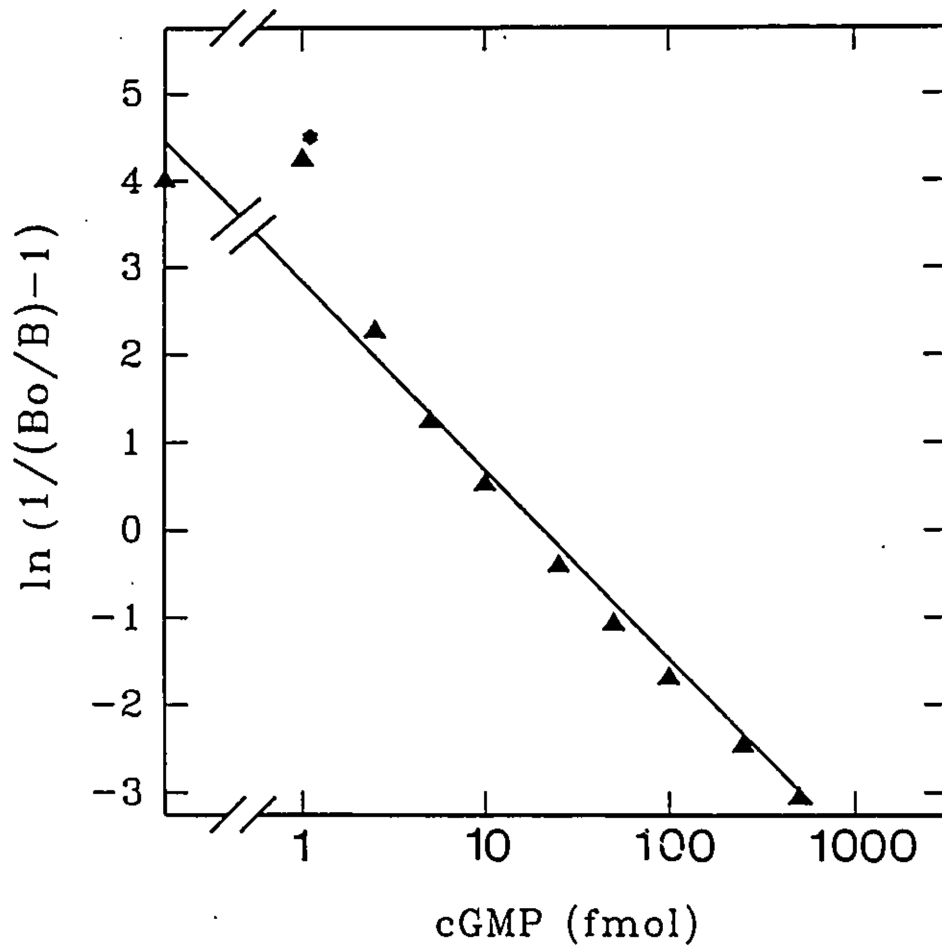
Each point (▲) represents the mean of two separate determinations. The line drawn through these points represents the best fit derived from a least squares regression analysis. Values not used in the regression analysis are indicated, *.



5
4
3
2
1
0
−1
−2
ln (1/(Bo/B)−1)
*
1
10
100
1000
cAMP (fmol)

Fig. 2.4. Representative standard curve for the radioimmunoassay of cGMP (Section 2.2.13.)

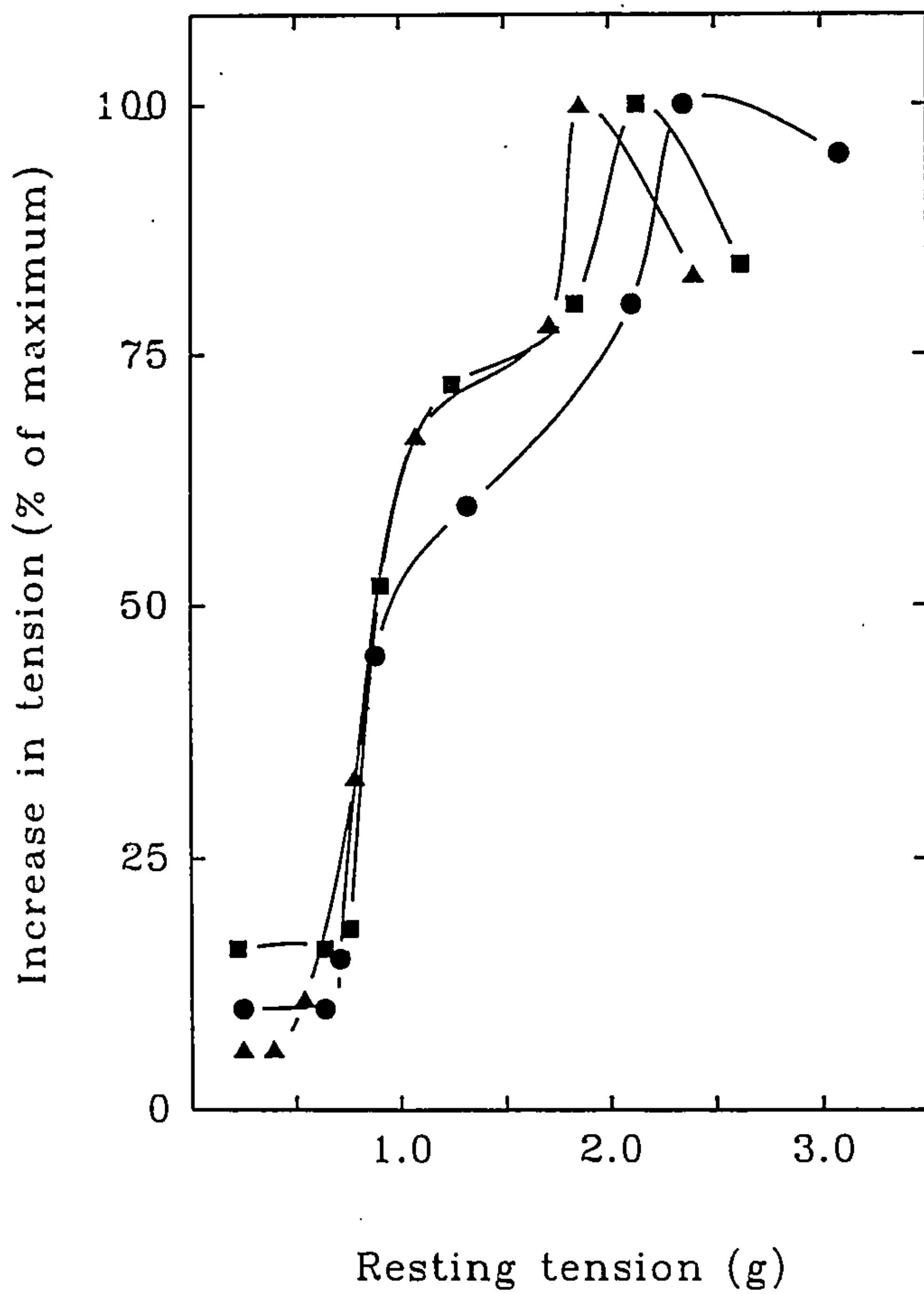
Each point (▲) represents the mean of two separate determinations. The line is derived from a least squares regression analysis. Values not used in the analysis are indicated, *.



5
4
3
2
1
0
-1
-2
-3
ln (1/(Bo/B)-1)
1
10
100
1000
cGMP (fmol)

Fig. 2.5. Effects of pre-load tension on the ability of rat aortic smooth muscle to contract in response to 30 mM KCl

The tension (g) to which equilibrated rat aortic rings were exposed was varied and the contractions that resulted from exposure of these rings to 30 mM KCl was determined. Curves represent the resting/active tension relationship for 3 different rings, all derived from one aorta.


100
75
50
25
0
1.0
2.0
3.0
Increase in tension (% of maximum)
Resting tension (g)

Chapter 3. *Inhibition of platelet function by nitrovasodilators and activators of adenylyl cyclase*

3.1. *Introduction*

3.1.1. *Effects of vasodilators on platelet and vascular smooth muscle function*

Certain nitrovasodilators, in particular SNP (Glusa *et al.*, 1974; Saxon and Kattlove, 1976) and SIN-1 (Nishikawa *et al.*, 1982), a metabolite of molsidomine, are potent inhibitors of platelet aggregation *in vitro*. Organic nitrates are much less effective but, at least in the case of nitroglycerin, can be activated by thiol compounds (Loscalzo, 1985; Gerzer *et al.*, 1988). There is also evidence that some nitrovasodilators can suppress platelet reactions *in vivo*, suggesting that an anti-platelet action may supplement their effects on vascular smooth muscle in the treatment of cardiovascular disease (Mehta and Mehta, 1980; Fielder, 1982; De Caterina *et al.*, 1984; Basista *et al.*, 1985).

Nitrovasodilators are believed to exert their effects via the release of nitric oxide, which stimulates the formation of cGMP by soluble guanylyl cyclase (Waldman and Murad, 1987). Consistent with this view, both SNP (Böhme *et al.*, 1978; Haslam *et al.*, 1980;

Mellion *et al.*, 1981) and SIN-1 (Nishikawa *et al.*, 1982; Gerzer *et al.*, 1988) activate platelet guanylyl cyclase and markedly increase cGMP in intact platelets. cGMP has been assumed to mediate vascular relaxation by stimulating the phosphorylation of critical protein substrates by cGMP-dependent protein kinase (Waldman and Murad, 1987). A similar mechanism has been proposed for platelets, in which the cGMP-dependent phosphorylation of specific but as yet unidentified proteins has also been described (Haslam *et al.*, 1980; Waldmann *et al.*, 1986). However, in this thesis and in previous studies in this laboratory (Haslam *et al.*, 1980) and in at least two others (Nishikawa *et al.*, 1982, Hawkins *et al.*, 1988) small increases in cAMP in human platelets incubated with either SNP or SIN-1 have been reported. Preliminary studies have suggested that this cAMP contributes to the inhibitory effects of SNP in human platelet aggregation (Haslam and Davidson, 1982).

Recent evidence has indicated that nitric oxide released from the vascular endothelium may play a physiological role in the regulation of vascular tone and interaction of the platelets with the vessel wall. Thus, EDRF which is released from the endothelium under basal conditions as well as at an accelerated rate in the presence of many pharmacological stimuli, has been shown to be very similar to nitric oxide (Palmer *et al.*, 1987; Ignarro *et al.*, 1987; Ignarro, 1989). EDRF not only relaxes vascular smooth muscle, but also inhibits platelet aggregation (Azuma *et al.*, 1986; Furlong *et al.*, 1987;

Busse *et al.*, 1987). In both cases, these effects are associated with the activation of guanylyl cyclase (Hawkins *et al.*, 1988; Ignarro, 1989). The formation of nitric oxide therefore represents a mechanism by which the vascular endothelium can inhibit platelet and smooth muscle responses additional to the formation of PGI_2, which exerts its effects through the receptor-mediated activation of adenylyl cyclase (Moncada, 1982).

Levin and colleagues (1982) reported that SNP and PGI_2 act synergistically to inhibit platelet aggregation. Thus, SNP markedly potentiated the inhibitory effects of PGI_2. More recently, synergism between the actions on platelets of PGI_2 and EDRF (Radomski *et al.*, 1987; Macdonald *et al.*, 1988) or of PGI_2 and organic nitrates (De Caterina *et al.*, 1988) has been demonstrated. Since PGI_2 and EDRF are likely to be generated simultaneously by stimulated vascular endothelium, a synergistic effect of these compounds on platelets could be of major physiological importance (Radomski *et al.*, 1987). Moreover, the actions of exogenous nitrovasodilators on platelets could be enhanced by endogenous PGI_2 formation (De Caterina *et al.*, 1988). This chapter presents the results of experiments designed to further our understanding of the molecular mechanism(s) responsible for these synergistic effects.

3.1.2. *Objectives*

As described in Section 3.1.1, activators of platelet adenylyl and guanylyl cyclases act in concert to cause a synergistic inhibition of platelet aggregation and degranulation. The objective of the first part of this thesis was to gain a better understanding of the molecular event(s) that are responsible for this effect. To achieve this, experiments were undertaken using a method that allowed the simultaneous measurement of changes in platelet cAMP and cGMP, as well as the inhibition of platelet function, that occurred during incubation of platelets with these compounds. Agents known to inhibit the effects of activators of adenylyl or guanylyl cyclases, that is 2',5'-dideoxyadenosine (DDA) and hemoglobin, respectively, and selective inhibitors of specific cyclic nucleotide PDEs (cilostamide and M&B 22,948) were used to establish the significance of correlations between changes in platelet cAMP and cGMP and the inhibition of platelet function.

3.2. *Results*

3.2.1. *Prelabelling technique for simultaneous measurement of [^{3}H]cAMP and [^{3}H]cGMP; purity of cyclic [^{3}H]nucleotides and comparison with radioimmunoassays*

Although the alumina-Dowex 50 two-column procedure (Jakobs *et*

al., 1976) has been successfully used to measure changes in [^{3}H]cAMP in rabbit platelets labelled with [^{3}H]adenine (Haslam and McClenaghan, 1981) or changes in [^{3}H]cGMP in human platelets labelled with [^{3}H]guanine (Davies *et al.*, 1976), these methods have not previously been combined or fully validated in rabbit platelets. The recoveries of cyclic [^{3}H]nucleotides were monitored by adding known amounts of pure [^{14}C]cAMP and [^{14}C]cGMP to the acidified incubation mixtures prior to purification of the labelled compounds. The final recoveries of both [^{3}H]cAMP and [^{3}H]cGMP averaged about 50% of the amounts present on acidification of the platelets. To normalize the results from different experiments, the [^{3}H]cAMP and [^{3}H]cGMP that accumulated in platelets after incubation with various additions were expressed as percentages of the [^{3}H]ATP and [^{3}H]GTP, respectively, that were present in an equal number of untreated platelets.

The purity of the [^{3}H]cAMP and [^{3}H]cGMP isolated by the two-column procedure was evaluated from a comparison of the ratios of ^{3}H/^{14}C present in these compounds before and after subsequent t.l.c. (Table 3.1.). Also, the ratios of ^{3}H/^{14}C present in these compounds before and after treatment with cyclic nucleotide phosphodiesterase were determined (Table 3.1). Almost all (89%) the basal [^{3}H]cAMP isolated behaved identically to authentic [^{14}C]cAMP on t.l.c. and 72% was found to co-chromatograph with AMP after phosphodiesterase treatment. Chromatography on t.l.c. showed that 39% of the trace [^{3}H]cGMP present in unstimulated platelets co-chromatographed with [^{14}C]cGMP and that

only 25% appeared to be authentic, based on treatment of the compound with the phosphodiesterase (Table 3.1.). However, both the increases in [^{3}H]cAMP caused by incubation of the platelets with PGE_1 and the increases in [^{3}H]cGMP caused by SNP co-chromatographed exclusively with the authentic ^{14}C-labelled cyclic nucleotides, both before and after phosphodiesterase treatments. Of particular interest was the finding that, although PGE_1 caused no increase in platelet [^{3}H]cGMP, SNP did increase platelet [^{3}H]cAMP (about 2.5-fold at 10 μM). The behaviour of this [^{3}H]cAMP on t.l.c. and its susceptibility to hydrolysis by PDE, proved that all this material was authentic [^{3}H]cAMP. SNP had an even more marked effect on the accumulation of [^{3}H]cAMP in the presence of a low concentration of PGE_1 (Table 3.1.). Again the material isolated under these conditions behaved identically to pure [^{14}C]cAMP (Table 3.1.).

Table 3.2. shows a direct comparison of the results obtained using prelabelling assays and radioimmunoassays to measure platelet cAMP and cGMP in parallel incubations of labelled and unlabelled platelets with SNP, PGE_1, or SNP and PGE_1. In this and two identical experiments, the specific activities of the [^{3}H]ATP and [^{3}H]GTP in the platelet metabolic (non-granular) pool were determined and the corresponding values for the pmol of [^{3}H]cAMP and [^{3}H]cGMP present were calculated. Under all experimental conditions used, these values were closely comparable with those obtained by radioimmunoassay, so demonstrating the validity of the prelabelling assays for cAMP and cGMP

in rabbit platelets (Table 3.2.). In particular, these results eliminate the remote possibility that some of the changes in [^{3}H]cAMP and [^{3}H]cGMP caused by SNP or PGE_1 might reflect changes in the specific radioactivities of the precursor nucleoside triphosphates. Table 3.2. also shows some novel results. Thus, incubation of rabbit platelets with SNP not only increased platelet cGMP, but also caused small but significant increases in platelet cAMP in the absence of PGE_1 and greatly potentiated the increases in cAMP observed in the presence of PGE_1. In the three experiments in which both assays were performed, addition of 1 μM SNP without PGE_1 increased platelet cAMP by 65 $\pm$ 7% and 93 $\pm$ 21%, as determined by the prelabelling assays and radioimmunoassays, respectively (mean values $\pm$ S.E.M.). These values are significantly different from the controls ($p < 0.05$) but not from each other. At the same time, addition of 1 μM SNP enhanced the *increases* in cAMP caused by PGE_1 by 453 $\pm$ 44% and 693 $\pm$ 92% in these two assays; both values are highly significant ($p < 0.005$). The biological importance and mechanism of these effects are the subject of the experiments described in the remainder of this chapter, in which the prelabelling assay alone was used. As shown in Table 3.2. and by the overall statistics given above, the standard errors observed in prelabelling assays were usually much smaller than those seen in the corresponding radioimmunoassays. The former assays were also much less expensive and time-consuming.

In the prelabelling experiments, labelling of [^{3}H]ATP and

$[^3H]$GTP averaged 2.6×10^6 d.p.m. and 1.6×10^6 d.p.m., respectively, in 2×10^8 platelets (the number used in most individual incubation mixtures). No changes in platelet $[^3H]$ATP or $[^3H]$GTP occurred during the course of experiments. Thus, in 3 experiments, $[^3H]$ATP values determined in control samples at the start ($2.8 \pm 0.0 \times 10^6$ d.p.m., mean ± S.E.M.) were not significantly different from values obtained at the end of the experiments ($2.9 \pm 0.2 \times 10^6$ d.p.m., mean ± S.E.M.). Similarly, in the same 3 experiments, values for $[^3H]$GTP obtained at the beginning ($1.8 \pm 0.0 \times 10^6$ d.p.m., mean ± S.E.M.) and the end ($1.7 \pm 0.0 \times 10^6$ d.p.m., mean ± S.E.M.), were not significantly different. Basal values for platelet $[^3H]$cAMP and $[^3H]$cGMP averaged 0.017 ± 0.001% and 0.012 ± 0.001% of the corresponding $[^3H]$nucleoside triphosphates (means ± S.E.M. from 25 experiments).

3.2.2. *Effects of different concentrations of SNP on rabbit platelets*

SNP caused concentration-dependent inhibitions of PAF-induced aggregation of rabbit platelets (Figs. 3.1. and 3.2a). Incubation of rabbit platelets with 0.1 μM, 1 μM or 10 μM SNP for 0.5 min inhibited aggregation by 14 ± 3%, 40 ± 9% or 71 ± 8%, respectively (means ± S.E.M. from 5 experiments). This compound also caused concentration-dependent increases in both $[^3H]$cAMP and $[^3H]$cGMP in rabbit platelets (Figs. 3.2b. and 3.3c.). Relative to the basal values,

the increases in [^{3}H]cGMP were by far the greater, from 0.011 ± 0.003% of platelet [^{3}H]GTP to 0.028 ± 0.005%, 0.093 ± 0.017% and 0.707 ± 0.176% of the platelet [^{3}H]GTP after 0.5 min incubation with 0.1 μM, 1.0 μM and 10 μM SNP, respectively (mean values ± S.E.M. from 10 experiments). At the same time, [^{3}H]cAMP increased from 0.021 ± 0.003% of platelet [^{3}H]ATP to 0.029 ± 0.004%, 0.044 ± 0.009% and 0.062 ± 0.015% of the [^{3}H]ATP (mean values ± S.E.M.). Despite some variation in cyclic [^{3}H]nucleotide levels between experiments, these increases in [^{3}H]cAMP were highly significant in each experiment (e.g. Table 3.2., Fig. 3.2b.) or when all the results were analysed by a paired *t* test ($p < 0.001$).

Analysis of the time course of cyclic [^{3}H]nucleotide accumulation caused by SNP showed that the maximum increase in [^{3}H]cGMP was reached earlier when higher concentrations of the nitrovasodilators were used and, with 10 μM SNP, declined rapidly after 30 s incubation (Fig. 3.3.). At the highest concentration tested (100 μM) SNP caused even larger increases in [^{3}H]cGMP in short incubations, but little further increase in [^{3}H]cAMP. The accumulation of [^{3}H]cAMP was shown to lag behind that of [^{3}H]cGMP and with 10 μM SNP continued to increase after [^{3}H]cGMP had begun to decline (Fig. 3.3d).

3.2.3. *Influence of DDA on the effects of SNP on rabbit platelets*

The results described in Section 3.2.2. suggested that the increases

in cGMP caused by SNP might stimulate the accumulation of cAMP and that cAMP might play a part in the inhibitory effects of SNP on platelet function. To test the latter possibility, DDA, an inhibitor of adenylyl cyclase that attenuates the effects on platelets of compounds that increase platelet cAMP was used (Haslam *et al.*, 1978). Although, the inhibition of PAF-induced platelet aggregation and degranulation by 0.1-10 μM SNP was not significantly decreased by 200 μM DDA under the experimental conditions used in the present study (Figs. 3.1. and 3.2a.), DDA did cause significant inhibitions of the accumulation of $[^3H]$cAMP ($p < 0.05$, paired *t* test). These varied from 62% with 0.1 μM SNP to 38% with 10 μM SNP (Fig. 3.2b.). DDA did not affect basal $[^3H]$cGMP, nor did it influence the increases in $[^3H]$cGMP caused by SNP (Fig. 3.2c.). These results suggest that cGMP is likely to mediate most of the inhibition of platelet function caused when rabbit platelets were incubated with SNP alone for 0.5 min.

3.2.4. *Synergistic effects of SNP and PGE_1 on rabbit platelets function*

Although incubation of rabbit platelets with SNP alone caused concentration-dependent inhibitions of PAF-induced platelet aggregation and degranulation, much more potent inhibitions were seen with 0.1 - 1.0 μM SNP in the presence of a concentration of PGE_1 chosen to have little effect alone (Figs. 3.1 and 3.2a). Thus, when added with 0.02 μM PGE_1, which inhibited platelet aggregation by only 6 $\pm$ 3%, the

inhibition caused by 0.1 μM SNP was increased from 14 ± 3% to 62 ± 6% (mean values ± S.E.M. from 5 experiments). This synergism was less conspicuous with 10 μM SNP, which caused a potent inhibition of platelet aggregation by itself (Fig. 3.2a).

3.2.5. *Effects of SNP and PGE_1 on rabbit platelet cyclic [^{3}H]nucleotides*

Synergistic interactions between PGE_1 and SNP with respect to [^{3}H]cAMP formation were observed with all SNP concentrations tested (Table 3.1., Table 3.2 and Fig. 3.2b). Incubation of rabbit platelets with 1 μM SNP, a concentration of the nitrovasodilator that increased [^{3}H]cAMP by 78%, potentiated the increases in [^{3}H]cAMP caused by 0.02 μM PGE_1 by 16-fold (mean from 4 experiments). The effects of these two vasodilators on the levels of [^{3}H]cAMP were clearly supra-additive ($p < 0.001$). The presence of PGE_1 did not, however, affect the increases in [^{3}H]cGMP caused by SNP (Table 3.1. Table 3.2 and Fig. 3.2c.). The relationship between the increases in [^{3}H]cAMP and [^{3}H]cGMP found in platelets incubated with different SNP concentrations in the presence and absence of 0.02 μM PGE_1 are shown in Fig. 3.4. In both cases, apparent saturation effects were observed; half-maximal increases in [^{3}H]cAMP were detected with increases in [^{3}H]cGMP equivalent to 0.05-0.1% of platelet [^{3}H]GTP. Again, this type of relationship is consistent with a role for cGMP in inducing the accumulation of cAMP.

3.2.6. *Effects of DDA on the synergistic effects of PGE_1 and SNP*

The above results suggested that the synergistic inhibitory effects of SNP and PGE_1 on platelet responses to PAF were mediated by an increased accumulation of $[^3H]$cAMP. In support of this hypothesis, addition of DDA was able to reduce the inhibition of aggregation caused by 0.1 μM SNP in the presence of 0.02 μM PGE_1 by about 50% (Fig. 3.1 and Fig. 3.2a.). This effect was associated with a marked decrease in $[^3H]$cAMP accumulation (Fig. 3.2b). Although DDA caused similar (about 65%) decreases in the $[^3H]$cAMP that accumulated when higher concentrations of SNP were added with PGE_1, its ability to suppress the inhibitory effects of these drug combinations decreased progressively as the SNP concentration increased (compare Figs. 3.2a. and Fig. 3.2b.). This may reflect a larger contribution of cGMP to the inhibitory effects of SNP added with PGE_1 at the higher SNP concentrations studied or, despite the effect of DDA, retention of sufficient $[^3H]$cAMP to inhibit function.

3.2.7. *Effects of SIN-1 on rabbit platelet aggregation*

To determine whether the ability of SNP to enhance the inhibition of aggregation caused by PGE_1 was a general property of nitrovasodilators, parallel experiments were carried out with SIN-1. Incubation of rabbit platelets with SIN-1 for 0.5 min caused concentration-dependent inhibition of PAF-induced aggregation.

Incubation with 10 μM SIN-1 inhibited aggregation by 32 ± 10%, whereas 100 μM SIN-1 caused an inhibition of 82 ± 4% (mean ± S.E.M. from 4 experiments). Together, SIN-1 and PGE_1 had a synergistic inhibitory effect on platelet aggregation and degranulation (Fig. 3.5a). At 10 μM, the nitrovasodilator potentiated the inhibition of aggregation caused by 0.02 μM PGE_1 from 26 ± 10% to 84 ± 12% (means ± S.E.M. from 4 experiments). The effect of the simultaneous incubation of the two compounds was supra-additive ($p < 0.005$). Synergism was not demonstrated with the higher concentration of SIN-1 tested, 100 μM, due to the substantial inhibition of function caused by this concentration of SIN-1 alone.

3.2.8. *Effects of SIN-1 and PGE_1 on cyclic $[^3H]$nucleotides in rabbit platelets*

When added alone, SIN-1 had effects almost identical to those obtained with lower concentrations of SNP (Fig. 3.5b. and Fig 3.5c.). This compound caused a marked increase in platelet $[^3H]$cGMP and a small, but significant, increase in $[^3H]$cAMP. Thus, incubation of prelabelled rabbit platelets with 10 μM or 100 μM SIN-1 for 0.5 min caused increases in $[^3H]$cGMP which amounted to 5 ± 1-fold and 26 ± 6-fold increases from basal values, respectively (means ± S.E.M. from 4 experiments, $p < 0.005$). The corresponding increases in $[^3H]$cAMP caused by 10 μM and 100 μM SIN-1 were 48 ± 8% and 124 ± 41% of basal, respectively. Although the latter effects were

small, the increases were significantly different from control (means $\pm$ S.E.M. from 4 experiments, $p < 0.05$). The synergistic inhibition of platelet function caused by incubation with both SIN-1 and PGE_1 was associated with a marked increase in the accumulation of $[^3H]$cAMP, as compared to the effects of either compound alone (Fig. 3.5b). Thus, in the presence of 10 μM SIN-1, the increase in $[^3H]$cAMP caused by 0.02 μM PGE_1 was potentiated 3-fold, from 0.025 $\pm$ 0.003% to 0.068 $\pm$ 0.010% of the $[^3H]$ATP (means $\pm$ S.E.M. from 4 experiments, $p < 0.005$). Similarly, 100 μM SIN-1, which increased basal $[^3H]$cAMP to 0.04 $\pm$ 0.01% of the $[^3H]$ATP alone, caused a 6-fold enhancement of the PGE_1-induced increase in $[^3H]$cAMP (mean $\pm$ S.E.M. from 3 experiments, $p < 0.02$). PGE_1 had no effect on SIN-1-induced increases in platelet $[^3H]$cGMP (Fig. 3.5c.).

3.2.9. *Effects of DDA on the synergistic actions of SIN-1 and PGE_1 on rabbit platelets*

DDA attenuated the synergistic inhibition of platelet function caused by these two compounds and in addition diminished the potentiation of the PGE_1-induced increase in $[^3H]$cAMP caused by 10 μM SIN-1 (Fig. 3.5a. and Fig. 3.5b.). DDA did not influence the inhibition of $[^{14}C]$5-HT release caused by addition of 10 μM SIN-1 (Fig. 3.5a.) or 100 μM SIN-1 alone. Thus, 100 μM SIN-1 inhibited release by 77 $\pm$ 1% in the absence and 80 $\pm$ 5% in the the presence of 200 μM DDA (mean $\pm$ S.E.M. from 3 experiments). These

values were not significantly different. Although DDA reduced the synergistic inhibition of $[^{14}C]$5-HT release caused by the simultaneous addition of 10 μM SIN-1 and 0.02 μM PGE_1 (Fig. 3.5a.), this agent did not reduce the inhibitions caused by the combined action of a higher concentration of SIN-1 and 0.02 μM PGE_1. Thus, the inhibitions caused by 100 μM SIN-1 and 0.02 μM PGE_1 were 99 $\pm$ 1% and 96 $\pm$ 4% in the absence and presence of DDA, respectively (mean $\pm$ S.E.M. from 3 experiments). The inability of DDA to decrease the effects of 100 μM SIN-1 in the presence of PGE_1 may reflect the large inhibitory effect of 100 μM SIN-1 alone. DDA inhibited the potentiation of the PGE_1-induced increase in $[^3H]$cAMP caused by 10 μM SIN-1 by 65 $\pm$ 3% (mean $\pm$ S.E.M. from 4 experiments) and that caused by 100 μM SIN-1 by 55 $\pm$ 7% (mean $\pm$ S.E.M. from 3 experiments). Again, DDA had no effect on basal or stimulated platelet $[^3H]$cGMP levels (Fig. 3.5c).

3.2.10. *Synergistic effects of nitrovasodilators and adenosine on rabbit platelets*

To establish whether the interaction between nitrovasodilators and PGE_1 was a general phenomenon associated with activation of adenylyl cyclase, or was specific to PGE_1, the effects of adenosine, another activator of platelet adenylyl cyclase (Haslam and Rosson, 1975), were compared to those to PGE_1. Addition of 1 μM adenosine alone increased platelet $[^3H]$cAMP by 84 $\pm$ 19% (mean $\pm$ S.E.M. from 3

experiments, $p < 0.01$), an effect similar to that caused by 1 μM SNP or 10 μM SIN-1. Adenosine had no effect on platelet [^{3}H]cGMP. However, when adenosine was incubated with either of the nitrovasodilators, it had a much more marked effect on [^{3}H]cAMP accumulation (Table 3.3.). These effects were comparable to those observed in the presence of both PGE_1 and SNP (Table 3.1. and Table 3.2.). Thus, 1 μM SNP or 10 μM SIN-1, concentrations of the nitrovasodilators that increased platelet [^{3}H]cAMP by 85 $\pm$ 43% and 41 $\pm$ 20%, respectively, potentiated the increase in [^{3}H]cAMP caused by 1 μM adenosine by 10 $\pm$ 3-fold and 11 $\pm$ 3-fold (Table 3.3.), respectively (means $\pm$ S.E.M. from 3 experiments). The effects of these compounds when incubated together were supra-additive ($p < 0.05$). Similarly, incubation of platelets with adenosine and either of the nitrovasodilators caused a synergistic inhibition of aggregation, comparable to that caused by the combined effects of PGE_1 and the nitrovasodilators (see Fig. 3.2a). Thus, 1 μM SNP and 10 μM SIN-1 increased the inhibition of PAF-induced aggregation caused by adenosine from 30% to 98% and 99%, respectively (means from 2 experiments). These findings suggest that nitrovasodilators may potentiate the effects on platelets of all compounds that activate platelet adenylyl cyclase.

3.2.11. *Effects of hemoglobin on the actions of the nitrovasodilators on rabbit platelets*

To investigate wether release of NO· by the nitrovasodilators was necessary for synergism between the nitrovasodilators and activators of adenylyl cyclase, the effects of hemoglobin, which is known to bind nitric oxide (Keilin and Hartree, 1937), were studied. Since aggregation recordings obtained in the presence of hemoglobin were difficult to interpret, the effects of this compound on the inhibition of platelet function by nitrovasodilators was studied by measuring the inhibition of the PAF-induced release of [^{14}C]5-HT. Pre-incubation of washed rabbit platelets with 10 μM hemoglobin for 0.5 min caused an almost complete reversal of the inhibition of [^{14}C]5-HT release caused by 1 μM SNP and 10 μM SIN-1 (Table 3.4.). Also, hemoglobin all but abolished the synergistic inhibition of platelet function caused by the simultaneous addition of the nitrovasodilators and the activators of adenylyl cyclase (Table 3.4.).

Hemoglobin also inhibited the increases in platelet [^{3}H]cGMP mediated by incubation of platelets with SNP and SIN-1 (Table 3.4. and Fig. 3.6.). In three experiments, the increase in [^{3}H]cGMP seen when platelets were incubated with 1 μM SNP was totally inhibited by pre-incubation with hemoglobin for 0.5 min. Similarly, hemoglobin totally inhibited the increases in [^{3}H]cGMP caused by the nitrovasodilator SIN-1 (10 μM). While hemoglobin had no effect

on the increase in platelet [^{3}H]cAMP caused by either PGE_1 or adenosine, it did inhibit the accumulation of [^{3}H]cAMP in response to the two nitrovasodilators (Table 3.4.). Thus, in three experiments the increase in [^{3}H]cAMP caused by 1 μM SNP or 10 μM SIN-1 was completely inhibited by pre-incubation of the platelets with hemoglobin. In one experiment, similar effects of hemoglobin on the actions of SIN-1 (10 μM) were observed (Table 3.4.).

The synergistic increases in [^{3}H]cAMP seen with the nitrovasodilators and the activators of adenylyl cyclase were likewise inhibited by hemoglobin (Table 3.4 and Fig. 3.6.). Thus, in the presence of 10 μM hemoglobin, the nitrovasodilator-induced potentiation of the increases in [^{3}H]cAMP, attributable to either PGE_1 or adenosine, were completely inhibited (Table 3.4 and Fig. 3.6). Hemoglobin was also effective when added 0.5 min after the nitrovasodilator and almost completely reversed the increases in cyclic [^{3}H]nucleotides, indicating that their maintenance required the continued presence of nitric oxide (Fig. 3.6). These results indicate that the formation and the action of nitric oxide was necessary for the increases in both [^{3}H]cGMP and [^{3}H]cAMP caused by nitrovasodilators and that these effects are freely reversible.

3.2.12. *Comparison of the effects of cilostamide and SNP in rabbit platelets*

Preliminary experiments showed that cilostamide, a selective inhibitor of the low K_m cAMP PDE of human and rabbit platelets (Hidaka *et al.*, 1979), increased platelet [^{3}H]cAMP in a concentration-dependent manner causing a near-maximal effect at 10 μM (Fig. 3.7). These increases in platelet [^{3}H]cAMP were comparable in magnitude with those observed in the presence of 0.1-10 μM SNP (Fig. 3.2b). Thus, incubation of prelabelled rabbit platelets with 1 μM cilostamide for 0.5 min increased basal [^{3}H]cAMP from 0.015 ± 0.001% of the platelet [^{3}H]ATP to 0.033 ± 0.004% of the [^{3}H]ATP (means ± S.E.M. from 5 experiments, $p < 0.005$). Moreover, cilostamide, like SNP, greatly potentiated the increase in [^{3}H]cAMP caused by a low concentration of PGE_1 (see Table 3.5 and Fig. 3.7). Thus, 1 μM cilostamide potentiated the increases in [^{3}H]cAMP caused by 0.02 μM PGE_1 by 3 ± 1-fold (mean ± S.E.M. from 5 experiments). Finally, in the presence of 10 μM cilostamide, SNP had no additional effect on platelet [^{3}H]cAMP, whether PGE_1 was included in the incubation medium or not (Table 3.5). This finding suggests that the increases in platelet [^{3}H]cAMP caused by SNP result from an inhibition of the cAMP PDE that is also the target of cilostamide. However, addition of 10 μM cilostamide increased the accumulation of [^{3}H]cAMP caused by a maximally effective concentration of SNP in the presence of PGE_1 (Table 3.5). The effects of SNP and

cilostamide differ in that the latter does not affect basal [^{3}H]cGMP levels in the platelet and, at 10 μM, only slightly increased the [^{3}H]cGMP that accumulated in response to SNP (Table 3.5). The above findings raise the possibility that cGMP, formed in response to addition of SNP, increases platelet cAMP by inhibiting the low K_m cAMP PDE that is also inhibited by cilostamide. Such an effect has been well-documented in studies with purified platelet cGI-PDE (Beavo, 1988), but has not previously been observed in intact cells.

3.2.13. *Effects of M&B 22,948 on cyclic [^{3}H]nucleotides in rabbit platelets*

To investigate the possible role of cGMP in the regulation of platelet cAMP breakdown, we studied the changes in platelet [^{3}H]cGMP and [^{3}H]cAMP caused by M&B 22,948, a selective inhibitor of cGMP PDE (Lugnier *et al.*, 1983). At 10 μM, the highest concentration tested, M&B 22,948 had no significant effect on [^{3}H]cGMP levels in resting platelets, suggesting that there is little turnover of cGMP under basal conditions (Fig. 3.8.). Platelet [^{3}H]cAMP was also unaffected by 10 μM M&B 22,948 alone (Fig. 3.8.). However, in the presence of SNP, 1-10 μM M&B 22,948 caused significant potentiation of the increases in both the [^{3}H]cGMP and [^{3}H]cAMP that accumulated in labelled platelets incubated with SNP (Fig. 3.8.). Thus, in the presence of 10 μM M&B 22,948, the increase in [^{3}H]cGMP caused by 1 μM SNP was potentiated by 233 ± 60% (mean ± S.E.M. from 3

experiments). Similarly, the SNP-induced accumulation of $[^3H]$cAMP was increased by 111 ± 64% (mean ± S.E.M. from 3 experiments). Both increases were significant in a paired *t*-test ($p < 0.05$). The increases in platelet $[^3H]$cAMP and $[^3H]$cGMP observed with all combinations of SNP and M&B 22,948 conformed to a single hyperbolic relationship, which yielded a linear Hill plot with a slope close to 1 (Fig. 3.9). A half-maximal increase in $[^3H]$cAMP required an increase in platelet $[^3H]$cGMP equivalent to 0.1% of platelet $[^3H]$GTP. Since the same relationship was found between the increases in platelet $[^3H]$cAMP and $[^3H]$cGMP caused by SNP, whether M&B 22,948 was present or not, M&B 22,948 is likely to have enhanced $[^3H]$cAMP accumulation solely by inhibiting cGMP PDE and so increasing the amount of cGMP present. Consistent with this, the potentiation of the SNP-induced increases in $[^3H]$cAMP caused by 10 μM M&B 22,948 were proportional to the ability of this compound to enhance the accumulation of $[^3H]$cGMP by the nitrovasodilator (Table 3.6). Also, M&B 22,948 potentiated the increase in $[^3H]$cAMP seen with 0.4 μM SNP by 38%, while only increasing the accumulation of $[^3H]$cAMP caused by 0.02 μM PGE_1 by 14% (means from 2 experiments), even though both compound had equivalent effects on $[^3H]$cAMP levels when used alone (Table 3.6). However, when higher concentrations of SNP or PGE_1 were used, M&B 22,948 had different effects (Table 3.6 and Fig. 3.10.). Thus, although 0.04 μM PGE_1 and 4 μM SNP had similar effects on platelet $[^3H]$cAMP when used alone, M&B 22,948 potentiated the increase caused by the higher concentration of PGE_1 by 33%, whereas it

augmented the accumulation of $[^3H]$cAMP caused by 4 μM SNP by only 10%. The basis for the latter effects is still not clear, although it may reflect non-specific effects of the PDE inhibitor, or an action of cGMP other than the inhibition of cGI-PDE. Overall, however, the results support the hypothesis that the increases in platelet $[^3H]$cAMP caused by SNP are secondary to increases in cGMP, which acts by inhibiting the low platelet cGI-PDE.

3.2.14. *Effects of SNP and PGE_1 on rat platelet aggregation*

The influence of these compounds on rat platelet aggregation was studied using thrombin, rather than PAF, as the aggregating agent. This was necessary because PAF does not aggregate rat platelets. The concentration of thrombin used (0.5 U/ml) was chosen because it caused almost complete aggregation of washed rat platelets within 1 min.

From results obtained in a preliminary aggregation experiment, 1 μM SNP was found to be an optimal concentration of this nitrovasodilator with which to study synergism. Thus, incubation of rat platelets with this concentration of SNP for 0.5 min caused 8 ± 2% inhibition of thrombin-induced aggregation (mean ± S.E.M. from 3 determinations). Incubation of rat platelets with PGE_1 (0.005 - 0.1 μM) for 0.5 min caused a concentration-dependent inhibition of aggregation. Simultaneous incubation of the platelets with the two compounds caused a potent supra-additive inhibition of thrombin-induced

aggregation (Table 3.7). The extent to which the action of PGE_1 onrat platelets was potentiated by SNP was comparable to the effects of this compound seen in washed rabbit platelets, incubated under similar conditions.

3.2.15. *Effects of SNP and PGE_1 on rat platelet cyclic [^{3}H]nucleotides*

Because [^{3}H]guanine was no longer available commercially, rat platelets were labelled with [^{3}H]guanosine and [^{3}H]adenine (Section 2.2.3.). Low concentrations of SNP caused increases in both [^{3}H]cGMP and [^{3}H]cAMP in prelabelled rat platelets (Table 3.8.). In percentage terms, the increases in [^{3}H]cGMP were much larger than those in [^{3}H]cAMP. Thus, incubation of rat platelets with 1 μM and 10 μM SNP for 0.5 min caused 7 $\pm$ 3-fold and 18 $\pm$ 4-fold increases in [^{3}H]cGMP, respectively (means $\pm$ S.E.M. from 3 experiments). The increases in [^{3}H]cAMP caused by 1 μM and 10 μM SNP amounted to 18 $\pm$ 5% and 38 $\pm$ 2% above the basal value, respectively (means $\pm$ S.E.M. from 3 experiments). These increases in [^{3}H]cGMP and [^{3}H]cAMP were both significant ($p < 0.05$).

Incubation of labelled rat platelets with PGE_1 caused concentration-dependent increases in [^{3}H]cAMP (Table 3.8.). Thus, 0.02 μM PGE_1 caused a 99 $\pm$ 13% increase in [^{3}H]cAMP above the basal value (mean $\pm$ S.E.M. from 3 experiments, $p < 0.005$). This

increase in [^{3}H]cAMP caused by PGE_1 was significantly potentiated by SNP (Table 3.8). Thus, when incubated with 1 μM SNP, which itself increased [^{3}H]cAMP only slightly, 0.02 μM PGE_1 increased platelet [^{3}H]cAMP by 204 ± 11% (mean ± S.E.M. from 3 experiments). The increase in [^{3}H]cAMP caused by these two compounds acting together was significantly supra-additive ($p < 0.01$).

3.2.16. *Comparison of the effects of cilostamide and SNP in rat platelets*

Cilostamide caused concentration-dependent increases in rat platelet [^{3}H]cAMP (Table 3.8). The maximum effect in platelets from this species was obtained with about 10 μM compound, a concentration that caused a 40 ± 3% increase in the level of this cyclic nucleotide (mean ± S.E.M. from 3 experiments). The effects of cilostamide or of SNP alone in rat platelets were similar, and comparable to the effects observed in rabbit platelets (Table 3.5). Both compounds also markedly potentiated the increases in [^{3}H]cAMP caused by PGE_1, but to different extents (Table 3.8). The difference between the maximum effects of these two compounds was larger in rat than rabbit platelets. (compare Tables 3.8. and Table 3.5). Whereas 10 μM SNP caused a 1.9-fold potentiation of the increase in [^{3}H]cAMP induced by 0.02 μM PGE_1, a concentration of cilostamide (1.0 μM) that gave the same increase in [^{3}H]cAMP as SNP when used alone, potentiated the action of PGE_1 by 4.1-fold (means ± S.E.M. from 3 experiments). Cilostamide had no effect on either basal [^{3}H]cGMP or the SNP-induced increases in [^{3}H]cGMP.

3.3. *Discussion*

3.3.1. *Cyclic nucleotide measurements in platelets*

By using both sensitive prelabelling techniques and radioimmunoassays, I have shown that the nitrovasodilator, SNP, causes small, at most three-fold, increases in cAMP in rabbit platelets, in addition to the expected large increases in cGMP. These two assays gave equivalent results, but the prelabelling assay, which was less subject to error, was used in the majority of experiments. The increases in platelet [^{3}H]cAMP caused by SNP were of a similar magnitude to those previously measured by radioimmunoassay in human and rabbit platelets (Haslam *et al.*, 1980; Hawkins *et al.*, 1988) and there is no reason to doubt their reality. Other workers, using radioimmunoassays, have failed to detect any change in platelet cAMP following incubation of platelets with either nitrovasodilators (Mellion *et al.*, 1981; Loscalzo, 1985; Morgan and Newby, 1989; Stamler *et al.*, 1989; Halbrügge *et al.*, 1989) or EDRF (Busse *et al.*, 1987; Alheid *et al.*, 1989). However, as was shown in this Chapter, the increases in platelet cGMP and cAMP caused by incubation of platelets with SNP were equivalent using both assays. Thus, the choice of method selected by these researchers cannot account for their inability to observe nitrovasodilator-induced increases in platelet cAMP levels. However, the experimental approach and the manner in which the cyclic nucleotide

measurements were carried out may be responsible for this discrepancy Thus, in some of these studies (Mellion *et al.*, 1981; Loscalzo, 1985), changes in platelet cyclic nucleotide levels were measured in PRP, without any attempt to allow for the cyclic nucleotide content of plasma. Since plasma contains relatively large amounts of both cAMP and cGMP, small changes in platelet cyclic nucleotide levels may have been difficult to measure. Only when cyclic nucleotides were purified before the radioimmunoassay, were significant increases in cAMP detected following incubation with nitrovasodilators (Haslam *et al.*, 1980; Hawkins *et al.*, 1988). The purification of the cyclic nucleotides may be necessary to reduce the extent to which substances present in platelet extracts interfere with the radioimmunoassays. In addition, in some studies (Alheid *et al.*, 1989; Morgan and Newby, 1989; Halbrügge *et al.*, 1989), the cAMP and cGMP were measured in platelet extracts without prior acetylation. Since the acetylation step is known to increase the sensitivity of these assays, it may have been impossible to detect small changes in cyclic nucleotide levels.

3.3.2. *Pharmacological impact on platelet function of the nitrovasodilator-induced increases in platelet [^{3}H]cAMP*

Although the increases in platelet [^{3}H]cAMP caused by both SNP or SIN-1 were always significant, the contributions of this cyclic [^{3}H]nucleotide to the nitrovasodilator-induced inhibitions of platelet

function were dependent on the experimental conditions. Thus, the attenuation of these increases in [^{3}H]cAMP by DDA, an inhibitor of adenylyl cyclase (Haslam *et al.*, 1978), without a corresponding effect on platelet aggregation, indicates that cAMP did not play a significant role in the inhibition of platelet function caused by SNP, when this compound alone was incubated with rabbit platelets. Presumably, cGMP mediated the inhibitory action of SNP under these conditions. This conclusion contrasts with preliminary results from similar experiments with human platelets in which DDA reduced the effects of SNP (Haslam and Davidson, 1982). The full reason for this species difference is not clear, though it presumably reflects a difference in the relative effectiveness of cAMP and cGMP as inhibitory second messengers in human and rabbit platelets. In the presence of a low PGE_1 concentration, the role of cAMP in the inhibition of platelet function was much clearer; SNP then caused very large additional increases in platelet cAMP, which with the lower concentrations of this compound, were associated with much more marked inhibitions of platelet aggregation and degranulation. Since DDA reduced both these increases in platelet [^{3}H]cAMP and the associated inhibitions of platelet function without affecting [^{3}H]cGMP levels, it follows that the synergistic effects of low concentrations of SNP and PGE_1 were mediated by cAMP. Similar results were obtained when SIN-1, the active metabolite of molsidomine, was used in place of SNP, suggesting that the observed phenomenon reflects a general property of nitrovasodilator drugs. In addition, results obtained in rat platelets (this Chapter),

as well as with human platelets (Haslam and Davidson, 1982), indicate that this potentiation of the effects of activators of adenylyl cyclase by activators of guanylyl cyclase may be a general mechanism, found in platelets from most species.

3.3.3. *Molecular basis for the nitrovasodilator-induced increases in platelet [^{3}H]cAMP*

In an earlier paper from Dr. Haslam's laboratory (Haslam *et al.*, 1980), it was suggested that the increases in platelet cAMP caused by SNP might result from the reported ability of guanylyl cyclase to make some use of ATP as a substrate (Mittal and Murad, 1977). There are three reasons which make such a mechanism unlikely. First, if both cyclic nucleotides were formed by the same enzyme, namely guanylyl cyclase, the time course of the formation of these two compounds would necessarily be identical. However, results from the present experiments demonstrated that the formation of [^{3}H]cAMP lagged behind that of [^{3}H]cGMP. The second reason relates to the effects of DDA, a "P" site inhibitor of platelet adenylyl cyclase (Londos and Wolff, 1977). Pre-incubation of the platelets with this compound, caused a marked reduction in the [^{3}H]cAMP that accumulated in response to treatment with either SNP or SIN-1, while leaving the increases in the levels of [^{3}H]cGMP unchanged. Thirdly, the relationship between [^{3}H]cAMP and [^{3}H]cGMP formation at different SNP concentrations was hyperbolic rather than linear. Moreover, the present studies suggest an entirely

different mechanism for the increases in platelet cAMP caused by nitrovasodilators. Since these compounds greatly enhanced the accumulation of [^{3}H]cAMP that occurred in the presence of activators of adenylyl cyclase, such as PGE_1 or adenosine, but caused no further increase in the presence of cilostamide, an inhibitor of platelet cAMP PDE (Hidaka *et al.*, 1979), they must act by inhibiting cAMP breakdown and not by promoting cAMP formation. Presumably, this reflects a direct or indirect inhibition of the low K_m cAMP PDE, that is the target of cilostamide and accounts for most of cAMP breakdown in both human and rabbit platelets (Beavo, 1988; Hidaka *et al.*, 1979). A proteolyzed form of this enzyme has been purified from human platelets (Grant and Colman, 1984) and was subsequently shown to be related immunologically to a low K_m cAMP PDE from bovine heart (Harrison *et al.*, 1986). In both instances, the purified enzyme was found to be inhibited by submicromolar concentrations of cGMP (Grant and Colman, 1984; Harrison *et al.*, 1986) and this enzyme has now been designated the cGI-PDE (see Section 1.14.3. and Beavo, 1990). These findings suggest that increases in platelet cAMP caused by nitrovasodilators might be mediated indirectly by cGMP. A number of the experiments described in this Chapter support this hypothesis. Thus, addition of hemoglobin, which binds nitric oxide released by nitrovasodilators and so blocks the activation of guanylyl cyclase by these compounds (Waldman and Murad, 1987), prevented the accumulation of *both* cGMP and cAMP. This shows that SNP and SIN-1 do not directly inhibit cAMP PDE but does not eliminate the formal possibility that nitric oxide does do so. The

effects of a selective inhibitor of platelet cGMP PDE, M&B 22,948 (Lugnier *et al.*, 1983) were more convincing. The experiments show that M&B 22,948 enhanced the accumulation of [^{3}H]cAMP caused by low SNP concentrations although, in contrast to cilostamide, it did not affect basal platelet [^{3}H]cAMP levels. Moreover, the relationship between the [^{3}H]cGMP and [^{3}H]cAMP accumulating in response to increasing SNP concentrations was unaffected by addition of M&B 22,948. These observations are most simply explained if [^{3}H]cAMP accumulation is a function of that of [^{3}H]cGMP. Indeed, the hyperbolic relationship observed suggests that cGMP acts at a site that is saturable at low concentrations of this cyclic nucleotide. Thus, a half-maximal effect of [^{3}H]cGMP on [^{3}H]cAMP accumulation was seen with an increase in the former equivalent to only about 0.1% of the [^{3}H]GTP present, which corresponds to some 12 pmol of cGMP/10^9 platelets (from Table 3.2.). These results provide the first evidence that cGMP inhibits cAMP breakdown in intact platelets and confirms speculations that the cGI-PDE might represent a target through which cGMP mediates some of its biological effects (Grant and Colman, 1984; Beavo, 1988; Walter, 1989). Recent studies have shown that the cGI-PDE from human platelets can be stimulated by cAMP-dependent phosphorylation (Macphee *et al.*, 1988; Grant *et al.*, 1988). However, the results of experiments described in this Chapter cast some doubt on the importance of this effect in intact rabbit platelets. Thus, the

markedly synergistic effects of SNP and PGE_1 on $[^3H]$cAMP accumulation indicate that inhibition of cAMP PDE activity by cGMP is the dominant regulatory mechanism. Since a similar synergism is seen in human platelets (Haslam and Davidson, 1982), the same conclusion applies in that species.

The findings discussed in this Chapter are complementary to indications that cGMP may enhance cAMP breakdown by a cGS-PDE in some cell types (see Section 1.14.2.; Beavo, 1988; Hartzell and Fischmeister, 1986). In fact, a possible interpretation of the observation that the maximum potentiation of the PGE_1-induced increase in $[^3H]$cAMP was less with SNP than with cilostamide in both rabbit and rat platelets, is that both cGMP-mediated inhibition and cGMP-mediated stimulation of cAMP breakdown could be components of the SNP response. Consistent with this, a cGS-PDE was recently purified from human platelets (Grant *et al.*, 1990). The enzyme hydrolysed both cAMP and cGMP, but the rate of hydrolysis of cAMP was stimulated by cGMP (0.1 μM - 10 μM). Since cilostamide does not increase cGMP, this second component would not be expected to participate in the effect of this compound. However, this hypothesis implies that cilostamide also inhibits the platelet cGS-PDE. Although such an effect has been reported *in vitro* with partially purified enzyme from human platelets (Hidaka *et al.*, 1979) and calf liver (Yamamoto *et al.*, 1983), the K_i values for these effects were relatively high (about 15 μM in each case). The effect of cilostamide on the enzyme purified by Grant *et al.* (1990)

was not reported. Alternatively, a mechanism involving activation of the cGI-PDE, by a cGMP-dependent phosphorylation reaction could account for the difference, since cilostamide does not affect cGMP levels. Further experiments will be required in order to address these questions.

Another possible mechanism by which cAMP and cGMP might interact to bring about synergistic inhibitory effects on platelet function, could be through the cGMP-PK. The two cGMP-binding sites of the cGMP-PK display very different binding affinities for the two cyclic nucleotides (reviewed by Beebe and Corbin, 1986). In addition, binding of cAMP to one site (Site 1) has been shown to cause a significant increase in the affinity of the second site for cGMP (Beebe and Corbin, 1986). Thus, in addition to an effect of cGMP on cAMP degradation, the cyclic nucleotides may interact at the level of the cGMP-PK. Consistent with this, Lincoln *et al.* (1990) have recently demonstrated, using cultured rat aortic VSMC, that the cGMP-PK may be responsible for some of the effects caused by increases in cAMP.

3.3.4. *Physiological significance of the interaction between activators of adenylyl and guanylyl cyclases*

PGE_1 can be regarded as a stable analogue of PGI_2, since both compounds stimulate platelet adenylyl cyclase through the same receptor (Moncada, 1982). It is PGI_2, synthesized by the vascular endothelium,

which is likely to be one of the most important local inhibitors of platelet aggregation under physiological conditions (Moncada, 1982; Haslam and McClenaghan, 1981). Nitric oxide, either released from nitrovasodilator drugs (Waldman and Murad, 1987) or generated endogenously by the vascular endothelium as the major component of EDRF (Palmer *et al.*, 1987; Ignarro *et al.*, 1987; Ignarro, 1989), also appears to decrease platelet responsiveness *in vivo* (Mehta and Mehta, 1980; Fielder, 1982; De Caterina *et al.*, 1984; Basista *et al.*, 1985; Bhardwaj *et al.*, 1988; Hogan et al, 1988). It follows that since both PGI_2 and nitrovasodilators (Levin *et al.*, 1982; De Caterina *et al.*, 1988) and PGI_2 and EDRF (Radomski *et al.*, 1987; Macdonald *et al.*, 1988) act synergistically to inhibit platelet aggregation, the cAMP-dependent mechanism that I have described may have important effects on platelet function, both enhancing the antithrombotic action of nitrovasodilator drugs and providing a basis for the physiological limitation of the growth of hemostatic plugs at sites of vascular injury. A similar mechanism could operate in other cells exposed to both nitric oxide and an activator of adenylyl cyclase, provided cAMP breakdown depends largely on the cGI-PDE, and that the effect of cGMP on inhibition of cAMP breakdown is the predominant effect. An important question is whether or not this is the case in vascular smooth muscle, since PGI_2 and EDRF also have marked effects on VSMC. The limited evidence available prior to the work described in Chapter 3 of this thesis was equivocal; a synergistic effect of PGI_2 and EDRF on relaxation had been observed with pig coronary artery

(Shimokawa *et al.*, 1988) but no synergism between a PGI_2 analogue and SNP was seen with rabbit coeliac and mesenteric arteries (Lidbury *et al.*, 1988).

3.4. *Summary*

3.4.1. Changes in $[^3H]$cGMP and $[^3H]$cAMP which occur upon incubation of platelets with activators of guanylyl and adenylyl cyclases were measured using a rapid and sensitive prelabelling technique. The validity of results obtained with this method were confirmed by radioimmunoassay of the two cyclic nucleotides. The prelabelling method was faster, easier, less expensive and less prone to error that the corresponding radioimmunoassays.

3.4.2. Incubation of rabbit or rat platelets with 0.1 to 10 μM SNP caused concentration-dependent inhibitions of platelet function that were associated with large increases in $[^3H]$cGMP and smaller but significant increases in $[^3H]$cAMP.

3.4.3. In rabbit platelets, SIN-1, caused similar concentration-dependent inhibition of platelet function and also increased both $[^3H]$cGMP and $[^3H]$cAMP.

3.4.4. Addition to rabbit platelets of either SNP or SIN-1 with concentrations of PGE_1 or adenosine that had little effect alone, caused synergistic inhibition of platelet function and supra-additive increases in $[^3H]$cAMP.

3.4.5. The adenylyl cyclase inhibitor, DDA diminished the increases in [^{3}H]cAMP caused by SNP or SIN-1, both in the presence and absence of PGE_1. DDA also reduced the inhibition of platelet function caused by the nitrovasodilators in the presence but not the absence of PGE_1. These results suggest that although cGMP may mediate the inhibition of rabbit platelet function by high concentrations of nitrovasodilator alone, the synergistic interactions of lower concentrations of these compounds with PGE_1 depends on an enhanced accumulation of cAMP.

3.4.6. Hemoglobin, which binds nitric oxide, blocked or reversed the increases in both [^{3}H]cGMP and [^{3}H]cAMP in platelets caused by the nitrovasodilators added either alone or with activators of adenylyl cyclase.

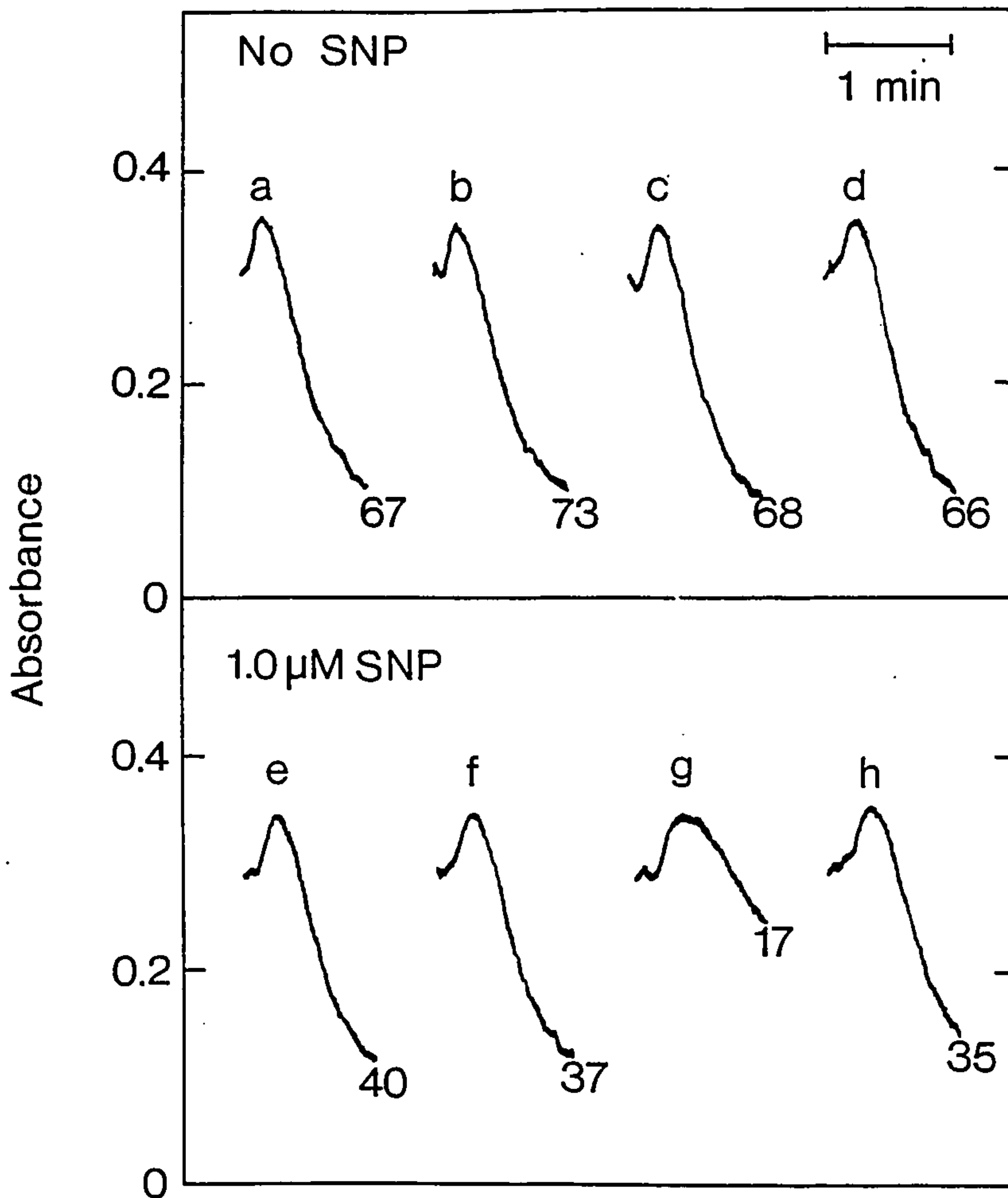
3.4.7. SNP had effects on [^{3}H]cAMP accumulation that were very similar to those of cilostamide, a selective inhibitor of platelet cGI-PDE. However, the maximum potentiation of the PGE_1-induced increases in [^{3}H]cAMP caused by SNP in rabbit or rat platelets was less than that caused by cilostamide. These results suggest that in intact platelets cGMP may both inhibit the breakdown of cAMP by the cGI-PDE and to a lesser extent potentiate its breakdown by a cGS-PDE.

3.4.9. M&B 22,948, a selective inhibitor of cGMP PDE, potentiated the increases in *both* [^{3}H]cGMP and [^{3}H]cAMP caused by SNP. A hyperbolic relationship was found between the increases in [^{3}H]cGMP and [^{3}H]cAMP caused by different concentrations of SNP; this relationship was not affected by addition of M&B 22,948.

3.4.10. The results strongly suggest that the synergistic inhibition of platelet function observed in the presence of activators of both adenylyl and guanylyl cyclases is mediated by cAMP, accumulating as a result of the inhibition of cAMP PDE activity by cGMP.

Fig. 3.1. Inhibition by SNP of PAF-induced platelet aggregation and $[^{14}C]$5-HT release; synergism between SNP and PGE_1 and reversal of the effects of PGE_1 by DDA

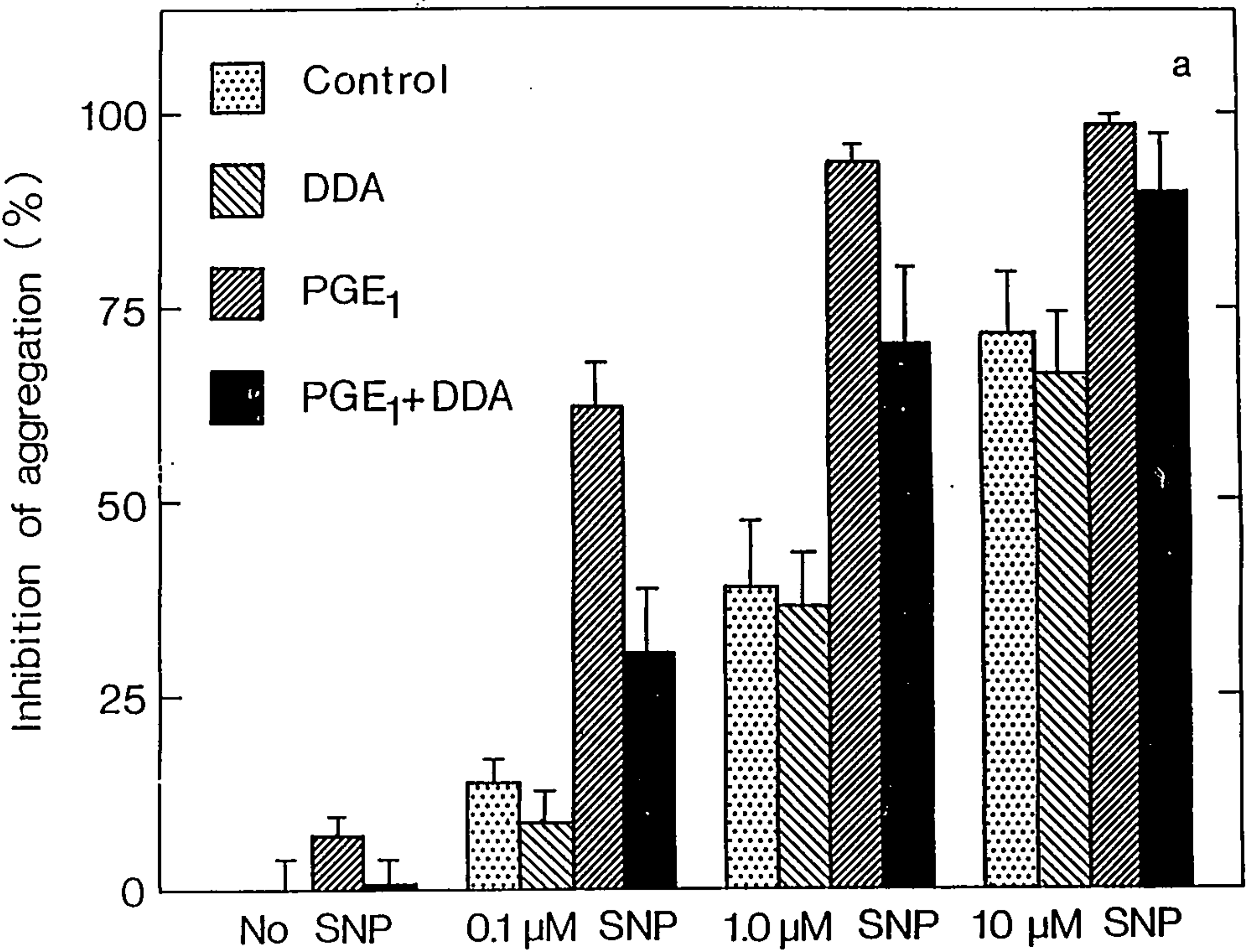
Labelled platelets containing $[^{14}C]$5-HT (2×10^8 platelets in a final vol. of 0.5 ml) were incubated at 37°C without (a - d) or with 1.0 μM SNP (e - h) and the following other additions: a and e, none; b and f, 200 μM DDA; c and g, 0.02 μM PGE_1; d and h, 0.02 μM PGE_1 and 200 μM DDA. DDA was added 0.5 min before any SNP or PGE_1 and the incubations were continued for a further 0.5 min. Platelet aggregation was then induced by the addition of 10 nM PAF with stirring. Aggregation was recorded for 1 min at which time the release of $[^{14}C]$5-HT was determined (see Section 2.2.5.). Each incubation was performed in triplicate; representative aggregation traces and the corresponding values for release of $[^{14}C]$5-HT (as % of total platelet ^{14}C) are shown.



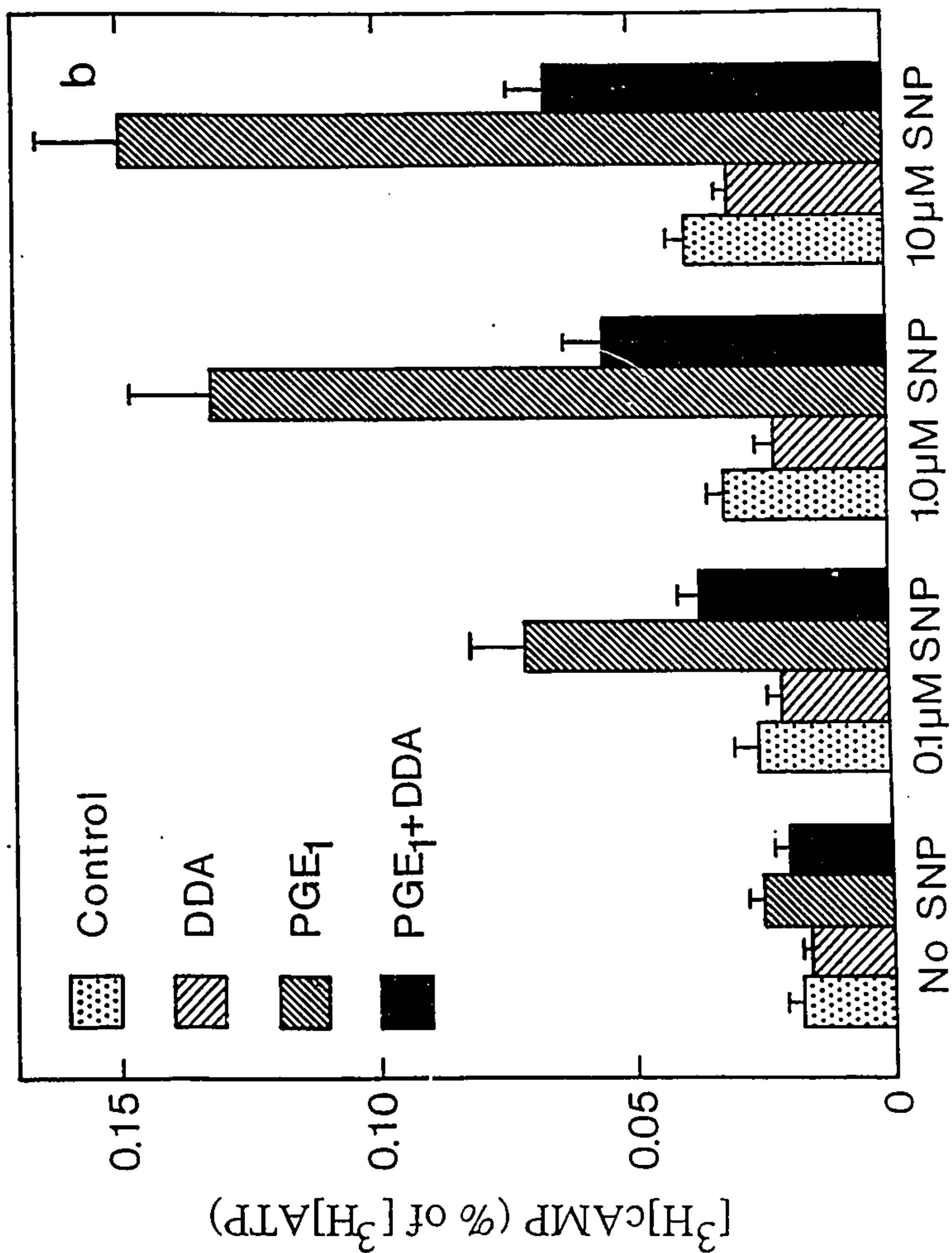
No SNP
1 min
1.0 μM SNP
Absorbance
0.4
0.2
0
a
b
c
d
e
f
g
h
67
73
68
66
40
37
17
35

Fig. 3.2. Effects of different concentrations of SNP on platelet aggregation, $[^3H]$cAMP and $[^3H]$cGMP in the absence and presence of DDA and PGE_1

Platelets were labelled with $[^3H]$guanine and $[^3H]$adenine (see Section 2.2.3.). Samples of the suspension were incubated at 37°C for 0.5 min with SNP (0-10 μM) in the absence or presence of 0.02 μM PGE_1, after an initial 0.5 min incubation with 200 μM DDA or vehicle, as indicated. After these incubations, part of each sample (0.5 ml, 2 x 10^8 platelets) was transferred to an aggregometer tube containing PAF (final concentration 10 nM) and the platelets were stirred for 0.5 min. (a) Aggregation was recorded and the percent inhibition determined from the changes in absorbance after 0.5 min in the presence and absence of the compounds studied. Values are means ± S.E.M. from 5 separate experiments with different platelet preparations. (b and c) After the above incubations, another part of each sample (0.5 ml, 2 x 10^8 platelets) was extracted with trichloroacetic acid. $[^3H]$cAMP (b) and $[^3H]$cGMP (c) were isolated and expressed as percentages of the corresponding $[^3H]$nucleoside triphosphates; values are means ± S.E.M. from 4 and 5 separate experiments, respectively.



a
Control
DDA
PGE_1
PGE_1+DDA
Inhibition of aggregation (%)
0
25
50
75
100
No SNP
0.1 µM SNP
1.0 µM SNP
10 µM SNP



b
Control
DDA
PGE1
PGE1+DDA
[3H]cAMP (% of [3H]ATP)
0.15
0.10
0.05
0
No SNP
0.1μM SNP
1.0μM SNP
10μM SNP

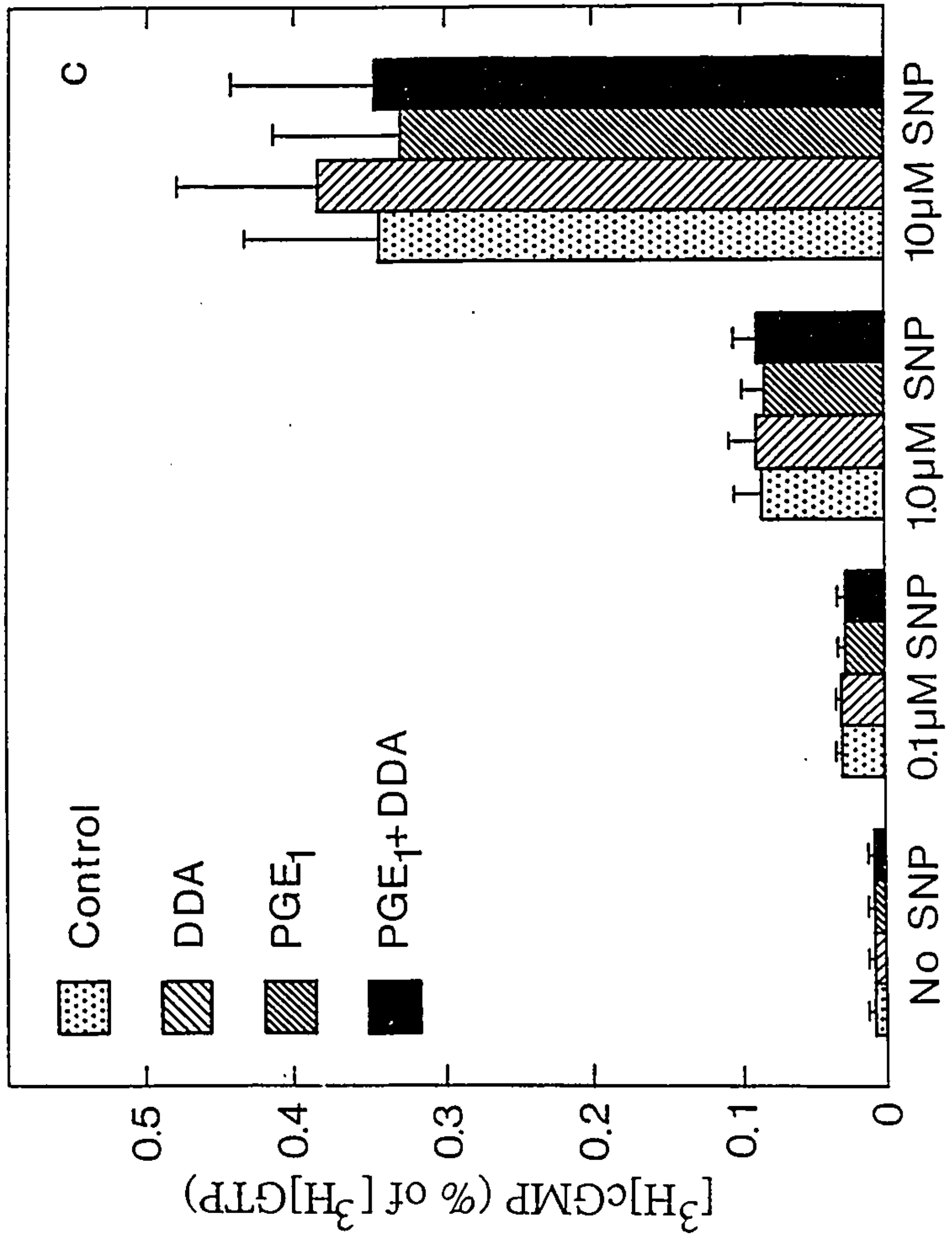

C
Control
DDA
PGE_1
PGE_1+DDA
No SNP
0.1µM SNP
1.0µM SNP
10µM SNP
0
0.1
0.2
0.3
0.4
0.5
[^{3}H]cGMP (% of [^{3}H]GTP)

Fig. 3.3. Time course of the SNP-induced accumulation of [^{3}H]cGMP and [^{3}H]cAMP in rabbit platelets

Platelets were isolated and labelled with [^{3}H]guanine and [^{3}H]adenine (see Section 2.2.3.). Samples of suspension containing 2 x 10^{8} labelled platelets were incubated at 37°C (final vol. 0.5 ml). Final concentrations of SNP were: a, none; b, 0.1 μM; c, 1.0 μM and d, 10.0 μM. The incubations were terminated after 0, 0.5, 1.0 or 3.0 min by addition of trichloroacetic acid (final concentration 5% (w/v)). [^{3}H]cGMP (▲) and [^{3}H]cAMP (●) were isolated and expressed as percentages of the total platelet [^{3}H]GTP and [^{3}H]ATP, respectively; values are means ± S.E.M. from 3 identical incubation mixtures.

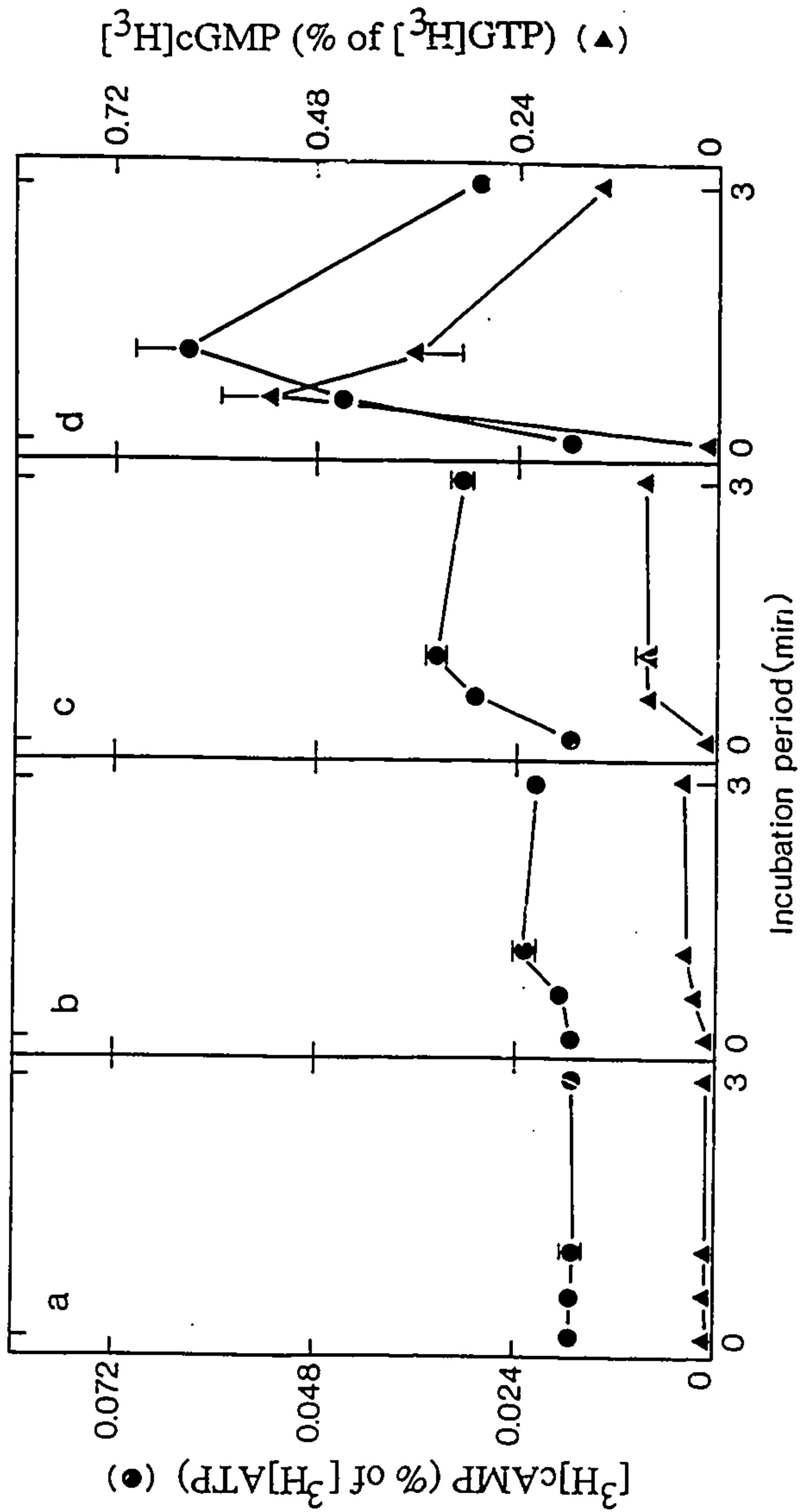
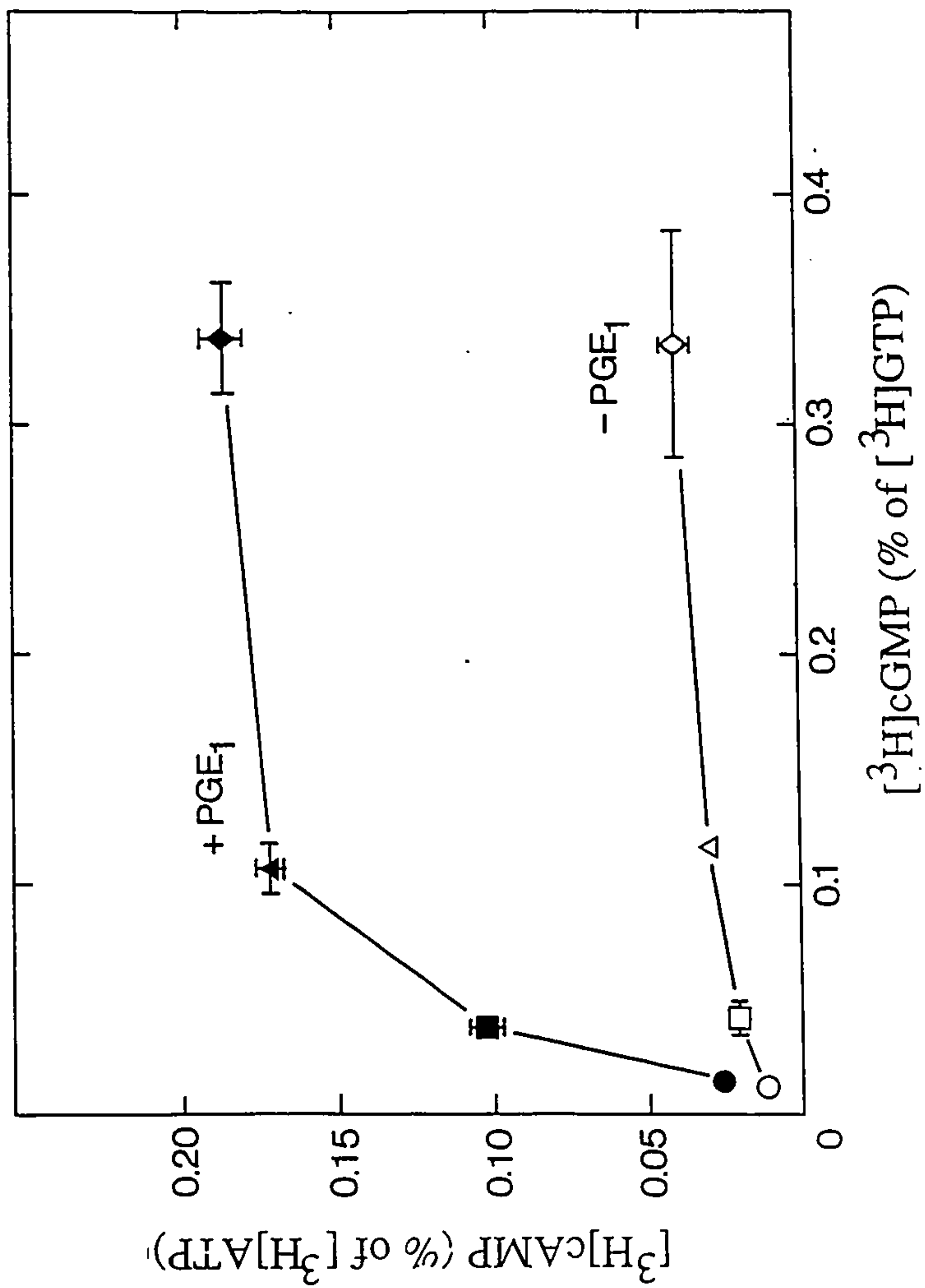

[3H]cGMP (% of [3H]GTP) (▲)
[3H]cAMP (% of [3H]ATP) (●)
Incubation period (min)
a
b
c
d
0.72
0.48
0.24
0
0.072
0.048
0.024
0
0
3

Fig. 3.4. Relationships between the increases in [^{3}H]cGMP and [^{3}H]cAMP in rabbit platelets caused by addition of SNP in the absence and presence of PGE_1

Platelets were labelled with [^{3}H]guanine and [^{3}H]adenine (see Section 2.2.3.). Samples of the suspension of labelled platelets were incubated for 0.5 min at 37° with no SNP (○ , ●), 0.1 μM SNP (□ , ■), 1.0 μM SNP (△ , ▲) or 10 μM SNP (◇ , ◆) in the absence (open symbols) or presence (closed symbols) of 0.02 μM PGE_1. Incubations were then terminated by the addition of trichloroacetic acid. [^{3}H]cGMP and [^{3}H]cAMP were isolated and expressed as percentages of the corresponding [^{3}H]nucleoside triphosphates; values are means ± S.E.M. from 3 identical incubation mixtures.



+PGE$_1$
−PGE$_1$
0
0.05
0.10
0.15
0.20
0.1
0.2
0.3
0.4
[^{3}H]cAMP (% of [^{3}H]ATP)
[^{3}H]cGMP (% of [^{3}H]GTP)

Fig. 3.5. Effects of SIN-1 on release of rabbit platelet [^{14}C]5-HT and on platelet [^{3}H]cAMP and [^{3}H]cGMP; synergism between SIN-1 and PGE_1 and reversal of the effects of PGE_1 by DDA

Platelets were labelled with [^{3}H]guanine, [^{3}H]adenine and [^{14}C]5-HT (see Section 2.2.3.). Samples of the suspension were incubated at 37°C for 0.5 min with 10 µM SIN-1 in the absence or presence of 0.02 µM PGE_1, after an initial 0.5 min incubation with 200 µM DDA or vehicle, as indicated. a, After these incubations, part of each sample (0.5 ml, 2 x 10^8 platelets) was transferred to an aggregometer tube containing PAF (final concentration 10 nM) and the platelets were stirred for 0.5 min at which time release of [^{14}C]5-HT was measured (see Section 2.2.5.). The percent inhibition of release of [^{14}C]5-HT was determined with respect to controls containing PAF alone. Values are means ± S.E.M. from 3 identical incubation mixtures. b and c, After the above incubations, another part of each sample (0.5 ml, 2 x 10^8 platelets) was extracted with trichloroacetic acid. [^{3}H]cAMP (b) and [^{3}H]cGMP (c) were isolated and expressed as percentages of the corresponding [^{3}H]nucleoside triphosphates; values are means ± S.E.M. from 3 identical incubation mixtures.

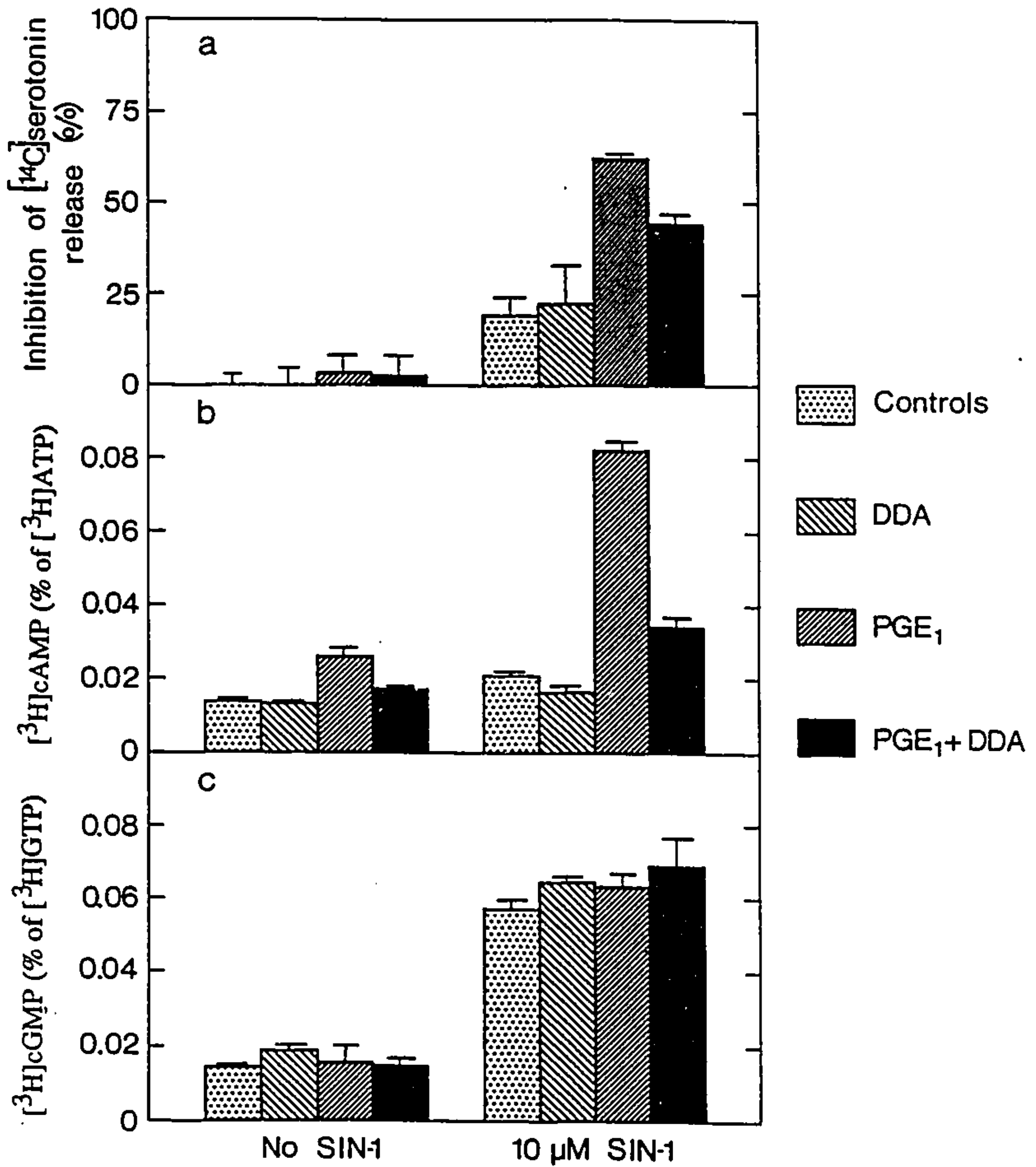
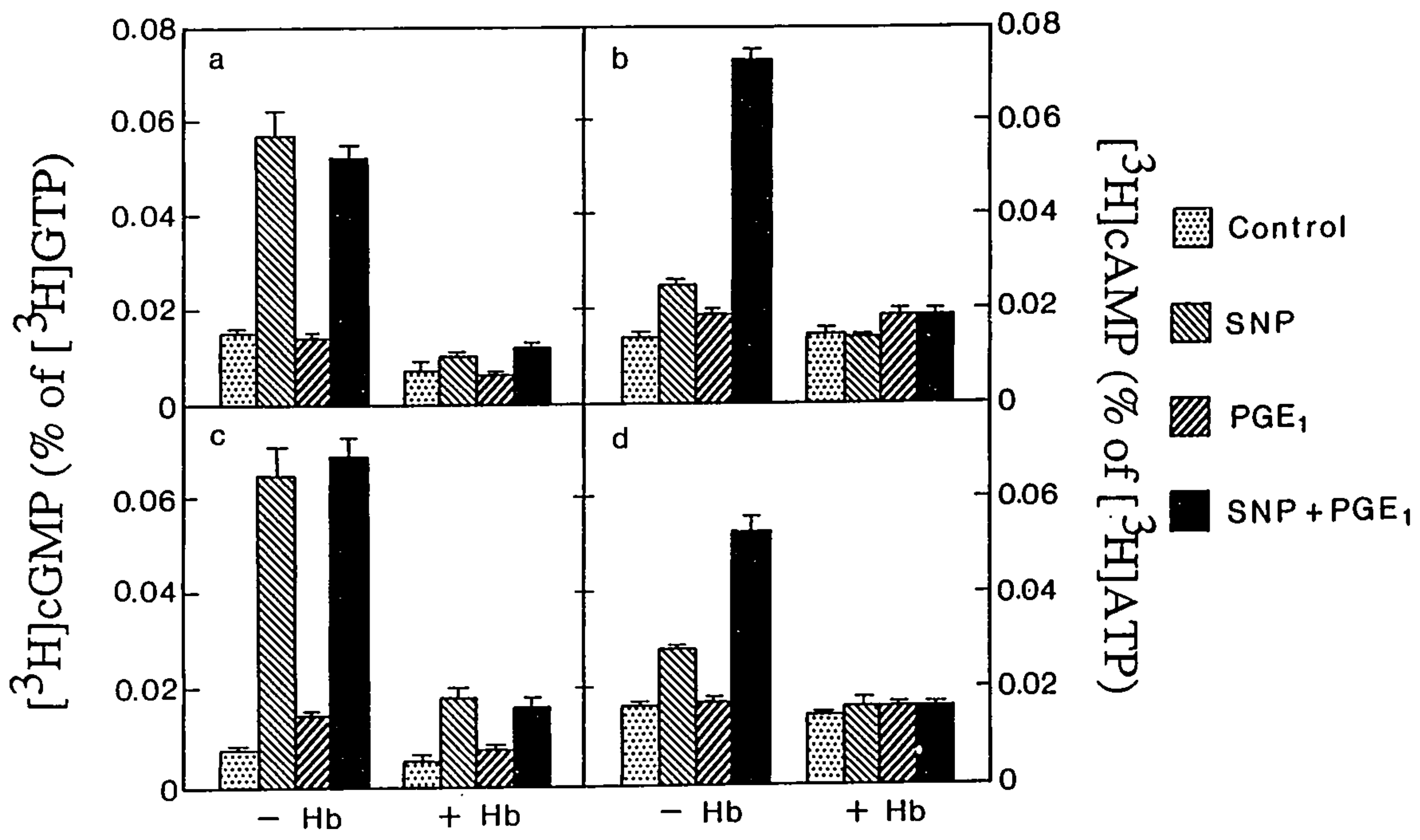

a
b
c
100
75
50
25
0
Inhibition of [14C]serotonin release (%)
0.08
0.06
0.04
0.02
0
[3H]cAMP (% of [3H]ATP)
[3H]cGMP (% of [3H]GTP)
No SIN-1
10 μM SIN-1
Controls
DDA
PGE1
PGE1+ DDA

Fig. 3.6. Inhibition by hemoglobin of the increases in $[^3H]$cGMP and $[^3H]$cAMP induced by SNP in the absence or presence of PGE_1

Platelets were labelled with $[^3H]$guanine and $[^3H]$adenine (see Section 2.2.3.). a and b, Samples of suspension (0.5 ml, 2 x 10^8 platelets) were incubated without hemoglobin or with 10 μM hemoglobin for 0.5 min at 37°C and then for a further 0.5 min with either no further addition, 1.0 μM SNP, 0.02 μM PGE_1 or 1.0 μM SNP and 0.02 μM PGE_1, as indicated. c and d, Samples of suspension (0.5 ml, 2 x 10^8 platelets) were incubated for 0.5 min at 37°C with SNP and/or PGE_1 (as above) but in the absence of hemoglobin. Following this incubation, 10 μM hemoglobin or an equal volume of 0.154 M NaCl was added to the samples, which were then incubated for a further 1.5 min before addition of trichloroacetic acid. $[^3H]$cGMP (a and c) and $[^3H]$cAMP (b and d) were determined; values are means $\pm$ S.E.M. from 3 identical incubation mixtures.



[3H]cGMP (% of [3H]GTP)
[3H]cAMP (% of [3H]ATP)
0.08
0.06
0.04
0.02
0
a
b
c
d
− Hb
+ Hb
Control
SNP
PGE1
SNP + PGE1

Fig. 3.7. Effects of cilostamide and PGE_1 on [^{3}H]cAMP in rabbit platelets

Platelets were washed and labelled with [^{3}H]guanine and [^{3}H]adenine (see Section 2.2.3.). Samples of suspension containing 2×10^8 platelets were incubated at 37°C for 0.5 min with the cilostamide concentrations indicated either in the presence (▽) or absence (●) of 0.02 μM PGE_1. Incubations were terminated by addition of trichloroacetic acid (5% (w/v), final). [^{3}H]cAMP is expressed as a percentage of the [^{3}H]ATP. Values are means ± S.E.M. from three identical incubation mixtures.

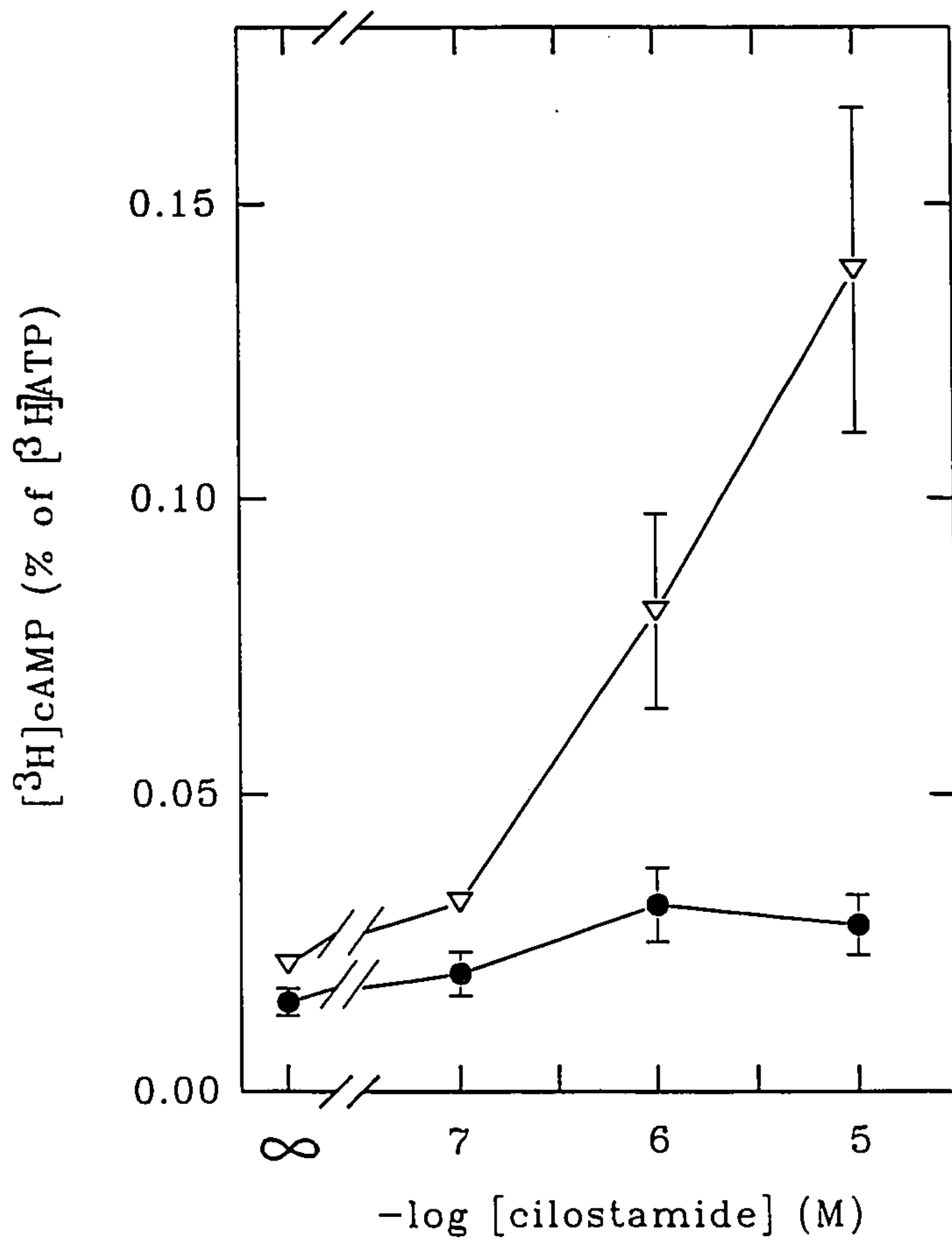

0.15
0.10
0.05
0.00
[3H]cAMP (% of [3 H]ATP)
∞
7
6
5
−log [cilostamide] (M)

Fig. 3.8. Effects of SNP and M&B 22,948 on [^{3}H]cGMP and [^{3}H]cAMP in rabbit platelets

Platelets were labelled with [^{3}H]guanine and [^{3}H]adenine (see Section 2.2.3.). Samples of the suspension (4 x 10^{8} platelets) were incubated for 0.5 min at 37°C with 0 - 4.0 μM SNP and the following additions (final vol. 1.0 ml): no M&B 22,948 (●); 2.5 μM M&B 22,948 (■); 10 μM M&B 22,948 (▲). All samples contained 0.2% (v/v) dimethyl sulphoxide. Incubations were then terminated by addition of trichloroacetic acid. [^{3}H]cGMP and [^{3}H]cAMP were isolated and expressed as percentages of the corresponding [^{3}H]nucleoside triphosphates; values are means $\pm$ S.E.M. from 3 identical incubation mixture.

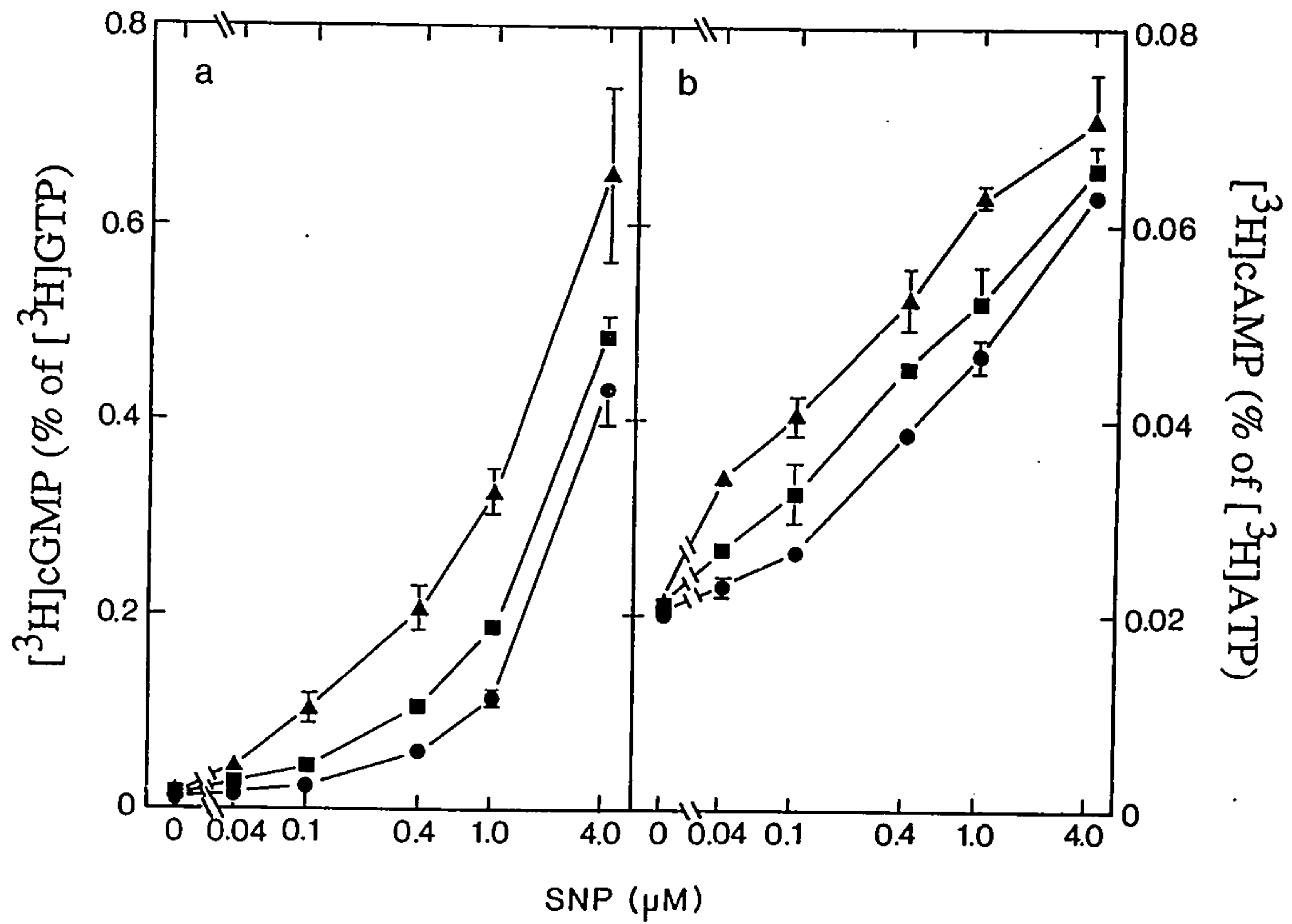

a
b
[3H]cGMP (% of [3H]GTP)
[3H]cAMP (% of [3H]ATP)
0.8
0.6
0.4
0.2
0
0.08
0.06
0.04
0.02
0
0 0.04 0.1 0.4 1.0 4.0
0 0.04 0.1 0.4 1.0 4.0
SNP (μM)

Fig. 3.9. Relationship between the increases in platelet [^{3}H]cGMP and [^{3}H]cAMP caused by SNP in the absence and presence of M&B 22,948

This figure is based on the results shown in Fig. 3.9. from incubations with 0.04 - 4 μM SNP, with the addition of values for [^{3}H]cGMP and [^{3}H]cAMP obtained in the same experiment in incubations with SNP and 1.0 μM M&B 22,948 (▼). The initial values of the cyclic [^{3}H]nucleotides are also shown (◊). The inset shows a Hill plot relating the increases in [^{3}H]cAMP (Δ y) to the increases in [^{3}H]cGMP (Δ x). Linear regression gave a Hill slope of 1.06 and a Δx intercept of -0.97 (r^2 = 0.984). The latter corresponds to a [^{3}H]cGMP concentration equivalent to 0.1% of platelet [^{3}H]GTP.

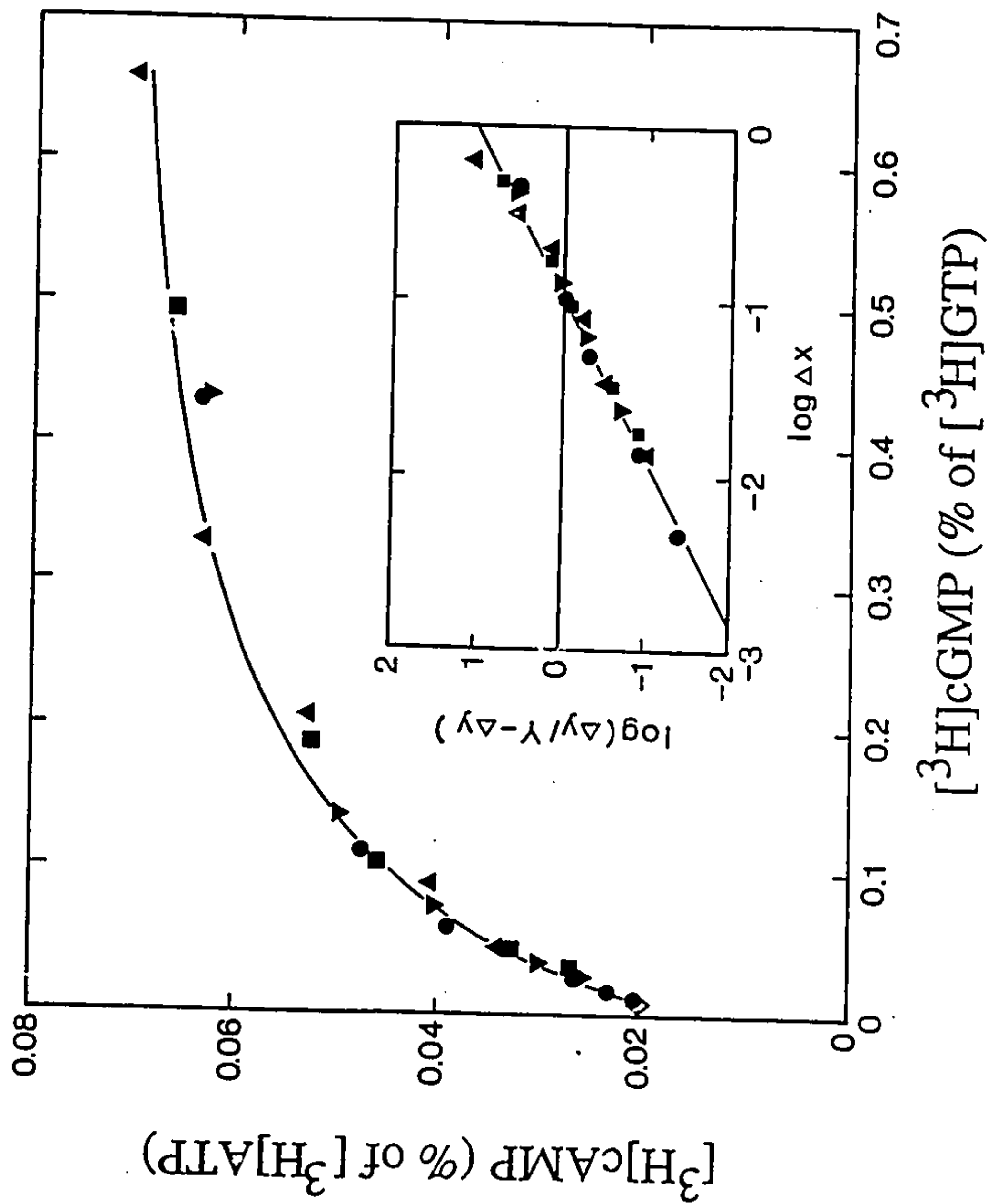

[3H]cAMP (% of [3H]ATP)
[3H]cGMP (% of [3H]GTP)
log Δx
log(Δy/Y−Δy)
0.08
0.06
0.04
0.02
0
0
0.1
0.2
0.3
0.4
0.5
0.6
0.7
2
1
0
−1
−2
−3
−2
−1
0

Fig. 3.10. Effects of M&B 22,948 on PGE_1-induced increases in [^{3}H]cAMP in rabbit platelets

Platelets were isolated and labelled with [^{3}H]adenine and [^{3}H]guanine (see Section 2.2.3.). Samples of the suspension (4 x 10^8 platelets) were incubated for 0.5 min at 37°C with the indicated concentrations of PGE_1, either alone (●) or in the presence of 2.5 μM (▼) or 10 μM M&B 22,948 (▲). Incubations were terminated by addition of ice-cold trichloroacetic acid (5% w/v final). [^{3}H]cAMP was isolated and is expressed as a percentage of the platelet [^{3}H]ATP. Values are means ± S.E.M. from three identical incubation mixtures.

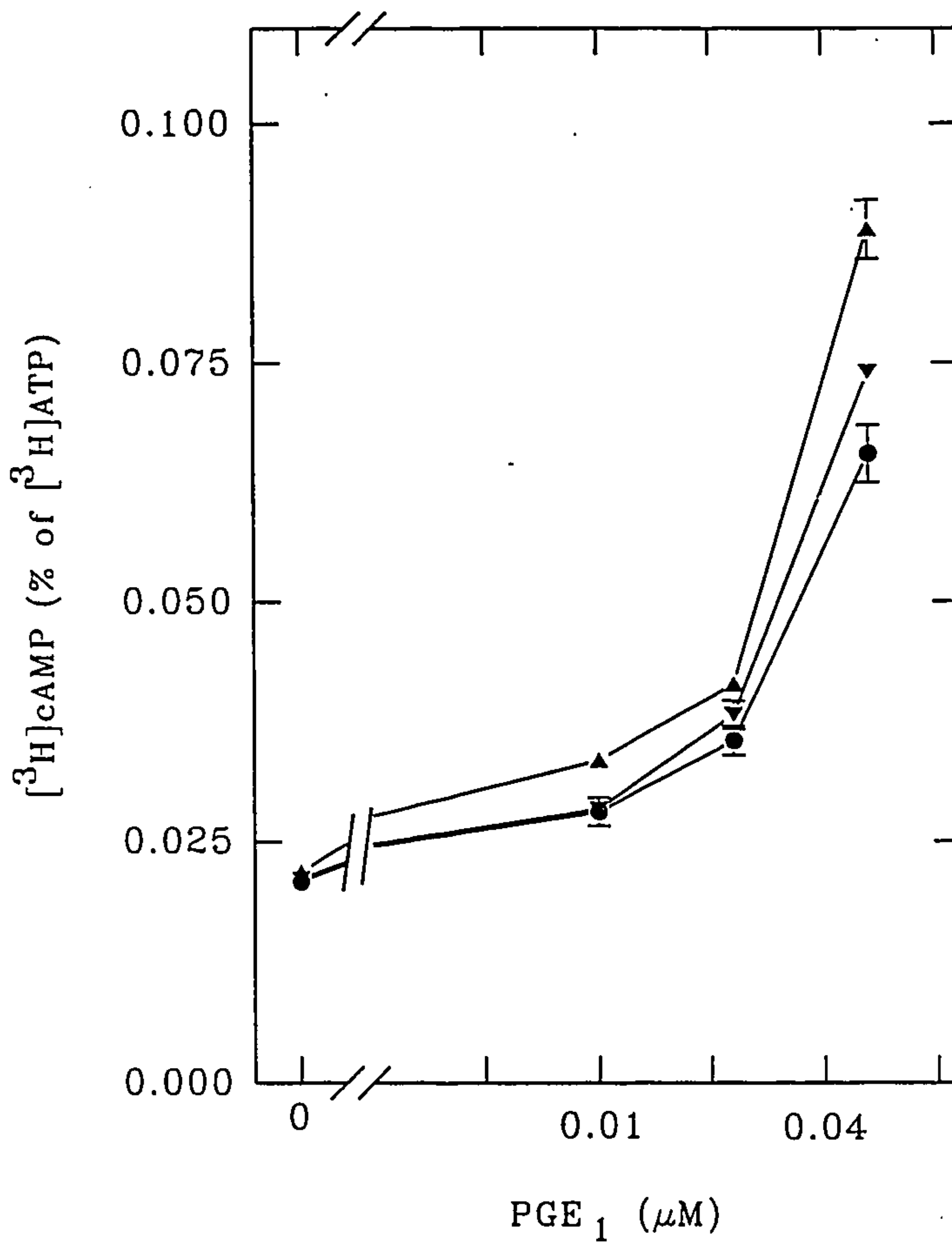

0.100
0.075
0.050
0.025
0.000
[3H]cAMP (% of [3H]ATP)
0
0.01
0.04
PGE 1 (μM)

Table 3.1. Purity of [^{3}H]cAMP and [^{3}H]cGMP isolated from prelabelled rabbit platelets by the two column procedure used in this study

Platelets were washed and labelled with [^{3}H]guanine and [^{3}H]adenine (see Section 2.2.1. and 2.2.3.). Samples of suspension containing 2 x 10^{8} labelled platelets were incubated for 0.5 min at 37°C with the additions indicated (final vol. 0.5 ml). Incubations were terminated by addition of trichloroacetic acid, followed by 660 d.p.m. of [^{14}C]cAMP and 710 d.p.m. of [^{14}C]cGMP. Labelled cAMP and cGMP were then isolated and separated by sequential chromatography on columns containing alumina and Dowex 50 resin (see Section 2.2.8). The cyclic nucleotides isolated from four identical incubation mixtures were counted for ^{3}H and ^{14}C; values for the total ^{3}H measured and the ^{3}H/^{14}C ratios are shown (means ± S.E.M.). The cAMP eluates from a further 16 identical incubation mixtures were pooled, dried by rotary evaporation and redissolved in 200 µl of 2 mM cAMP. In order to further assess the purity of the cyclic nucleotides eluted from the columns, a fraction (90 µl) of the 200 µl sample was incubated with bovine heart cyclic 3',5'-nucleotide PDE. This sample was added to a polypropylene tube containing 10 µl of a concentrated PDE assay mixture, which consisted of 10 mM TES (pH 7.5), 4 mM $MgSO_4$, 0.25 mM EDTA and 0.2 units of PDE/ml. The mixture was incubated for 60 min at 30°C after which the reaction was terminated by boiling. Following centrifugation, 20 µl of the reaction mixture was analysed by t.l.c. on cellulose and the 5'-nucleotide was eluted and counted. The purity of the cyclic nucleotides could be determined from a comparison of the ratio of ^{3}H/^{14}C of the 5'-AMP after treatment, to that of the fraction before the PDE treatment. This method was also used to purify 5'-GMP from the same incubation mixtures. Mean values for the ratios of ^{3}H/^{14}C after t.l.c. of both cyclic nucleotides are given.

Table 3.1.

Additions	Labelled cyclic nucleotide isolated	Amount of ^{3}H-labelled cyclic nucleotide d.p.m./sample	Ratio of $^{3}H/^{14}C$ before t.l.c. of cyclic nucleotide	Ratio of $^{3}H/^{14}C$ of 5'nucleotides after t.l.c.
None --	cAMP	405 ± 7	0.61 ± 0.01	0.44
	cGMP	314 ± 3	0.44 ± 0.00	0.11
PGE_1 (0.02 μM)	cAMP	487 ± 27	0.74 ± 0.04	0.73
	cGMP	278 ± 28	0.39 ± 0.04	0.37
PGE_1 (0.40 μM)	cAMP	13869 ± 409	20.98 ± 0.63	21.59
	cGMP	259 ± 32	0.37 ± 0.05	0.11
SNP(10.0 μM)	cAMP	1034 ± 57	1.57 ± 0.09	1.57
	cGMP	2314 ± 166	3.27 ± 0.23	3.47
PGE_1 + (0.02 μM) SNP (10.0 μM)	cAMP	3611 ± 170	5.46 ± 0.26	5.75
	cGMP	2258 ± 126	3.19 ± 0.18	3.18

PAGINATION ERROR.

ERREUR DE PAGINATION.

TEXT COMPLETE.

LE TEXTE EST COMPLET.

NATIONAL LIBRARY OF CANADA.

CANADIAN THESES SERVICE.

BIBLIOTHEQUE NATIONALE DU CANADA.

SERVICE DES THESES CANADIENNES.

Table 3.2. Comparison of values obtained for platelet cyclic nucleotides using prelabelling assays and radioimmunoassays

Washed platelets were incubated with ^{3}H-labelled or unlabelled guanine and adenine. The former platelets were used to measure changes in [^{3}H]cAMP and [^{3}H]cGMP by the prelabelling technique and the latter, changes in cAMP and cGMP mass by radioimmunoassay (see Section 2.2.13). In both instances, samples of platelet suspension (4×10^{8} platelets) were incubated for 0.5 min at 37°C with the additions indicated (final vol. 1 ml). Incubations were terminated by addition of trichloroacetic acid; ^{14}C-labelled cyclic nucleotides were added to monitor the recoveries of [^{3}H]cAMP and [^{3}H]cGMP in the prelabelling assays and ^{3}H-labelled cyclic nucleotides were added to monitor the recoveries of cAMP and cGMP prior to radioimmunoassay. Cyclic nucleotides were then isolated and assayed as described under Section 2.2.8. [^{3}H]cAMP and [^{3}H]cGMP determined in the prelabelling assays are expressed as percentages of the corresponding ^{3}H-labelled nucleoside triphosphates and as pmol/10^{9} platelets, calculated from the specific activities of the [^{3}H]ATP and [^{3}H]GTP recovered from the platelet cytosol (see Section 2.2.10). Platelet cAMP and cGMP determined by radioimmunoassay are also expressed as pmol/10^{9} platelets. All values are means ± S.E.M. from 4 identical incubation mixtures. Significant changes are shown: *, $p < 0.01$.

Table 3.2.

Additions	cAMP			cGMP		
	Prelabelling assay		Radioimmunoassay	Prelabelling assay		Radioimmunoassay
	% of [^{3}H]ATP	pmol/ 10^9 platelets	pmol/ 10^9 platelets	% of [^{3}H]GTP	pmol/ 10^9 platelets	pmol/ 10^9 platelets
None	0.014 ± 0.000	7.2 ± 0.1	5.9 ± 0.7	0.011 ± 0.001	1.3 ± 0.2	0.3 ± 0.3
SNP (1.0 μM)	0.021 ± 0.000	10.9 ± 0.2*	11.0 ± 0.9*	0.056 ± 0.004	6.6 ± 0.5*	8.9 ± 1.1*
SNP (10 μM)	0.026 ± 0.000	14.0 ± 0.2*	11.7 ± 0.5*	0.293 ± 0.031	34.7 ± 3.7*	28.6 ± 1.4*
PGE_1 (0.04 μM)	0.053 ± 0.002	27.8 ± 1.2*	20.9 ± 1.4*	0.009 ± 0.001	1.0 ± 0.1	1.5 ± 0.6
PGE_1 + SNP (0.04 μM) + (1.0 μM)	0.189 ± 0.003	99.7 ± 1.6*	103.3 ± 6.4*	0.047 ± 0.002	5.5 ± 0.2*	6.2 ± 1.3*

Table 3.3. Synergism between SNP and adenosine with respect to increases in platelet [^{3}H]cAMP but not [^{3}H]cGMP

Platelets were labelled with [^{3}H]guanine and [^{3}H]adenine (see Section 2.2.3.). Samples of suspension containing 2 x 10^8 platelets were incubated for 0.5 min at 37° with 1.0 μM SNP, 10 μM SIN-1 or 1.0 μM adenosine (AD) alone or in combination, as indicated (final vol. 0.5 ml). Incubations were terminated with trichloroacetic acid. [^{3}H]cGMP and [^{3}H]cAMP were isolated and each was expressed as a percentage of the corresponding [^{3}H]nucleoside triphosphate (see Section 2.2.10). Values are means ± S.E.M. from 4 identical incubation mixtures. Significant increases in cyclic [^{3}H]nucleotides are shown: * $p < 0.05$.

Additions	[^{3}H]cAMP (% of [^{3}H]ATP)	[^{3}H]cGMP (% of [^{3}H]GTP)
None	0.014 ± 0.001	0.007 ± 0.000
SNP	0.036 ± 0.001*	0.053 ± 0.006*
SIN-1	0.022 ± 0.001*	0.111 ± 0.016*
AD	0.031 ± 0.001*	0.010 ± 0.003
SNP + AD	0.276 ± 0.005*	0.037 ± 0.005*
SIN-1 + AD	0.143 ± 0.003*	0.100 ± 0.017*

Table 3.4. Effects of hemoglobin on the synergistic interactions between nitrovasodilators and activators of adenylyl cyclase

Platelets were labelled with [^{3}H]guanine and [^{3}H]adenine (see Section 2.2.3). Samples of suspension (1.2 ml, 4 x 10^{9} platelets/ml) were incubated with or without hemoglobin for 0.5 min at 37°C and then for a further 0.5 min with the indicated additions. Concentrations used were; PGE_1 (0.02 μM), adenosine (AD) (1 μM), SNP (1 μM), SIN-1 (10 μM). After these incubations, part of each samples (0.5 ml, 2 x 10^{8} platelets) was transferred to an aggregometer tube containing PAF (10 nM) and the platelets were stirred for 0.5 min at which time [^{14}C]5-HT release was measured (see Section 2.2.5.). The percent inhibition of release of [^{14}C]5-HT was determined with respect to controls containing PAF alone. Another part of each sample (0.5 ml, 2 x 10^{8} platelets) was extracted with trichloroacetic acid (5% w/v, final) and the cyclic [^{3}H]nucleotides isolated and expressed as percentages of the corresponding [^{3}H]nucleoside triphosphates. Values are means $\pm$ S.E.M. from three identical incubations. Significant increases from basal (* $p < 0.05$) and significant effects of hemoglobin (^{a} $p < 0.05$), are shown.

Table 3.4.

Additions	Hemoglobin	$[^3H]$cAMP (% of $[^3H]$ATP)	$[^3H]$cGMP (% of $[^3H]$GTP)	Inhibition of $[^{14}C]$5-HT release(%)
None	-	0.016 ± 0.001	0.021 ± 0.004	--
	+	0.015 ± 0.001	0.021 ± 0.005	4.0 ± 7.3
PGE_1	-	0.021 ± 0.002*	0.030 ± 0.005	5.3 ± 3.4
	+	0.020 ± 0.001*	0.018 ± 0.001	6.5 ± 8.1
AD	-	0.025 ± 0.002*	0.026 ± 0.003	4.3 ± 6.3
	+	0.020 ± 0.000*	0.019 ± 0.001	4.4 ± 5.1
SNP	-	0.028 ± 0.002*	0.094 ± 0.014*	65.7 ± 5.1*
	+	0.016 ± 0.001a	0.019 ± 0.002a	11.2 ± 3.9*a
SIN-1	-	0.026 ± 0.001*	0.141 ± 0.010*	57.5 ± 9.1*
	+	0.014 ± 0.001a	0.014 ± 0.002a	17.4 ± 7.3a
PGE_1 + SNP	-	0.073 ± 0.001*	0.068 ± 0.002*	95.8 ± 1.9*
	+	0.020 ± 0.000*a	0.021 ± 0.001a	16.9 ± 8.3a
PGE_1 + SIN-1	-	0.127 ± 0.003*	0.288 ± 0.016*	92.8 ± 0.7*
	+	0.025 ± 0.002*a	0.035 ± 0.005a	19.4 ± 10.1a
AD + SNP	-	0.124 ± 0.000*	0.079 ± 0.002*	85.5 ± 6.6*
	+	0.023 ± 0.001*a	0.017 ± 0.004a	16.9 ± 8.3a
AD +SIN-1	-	0.183 ± 0.005*	0.179 ± 0.011*	91.2 ± 1.2*
	+	0.031 ± 0.000*a	0.018 ± 0.003a	33.0 ± 17.8a

Table 3.5. Comparison of the effects of SNP and cilostamide on platelet cyclic [^{3}H]nucleotides

Platelets were washed and labelled with [^{3}H]guanine and [^{3}H]adenine (see Section 2.2.3.). Samples of suspension containing 4 x 10^{8} platelets were incubated for 0.5 min at 37°C with 1.0 μM SNP, 10 μM cilostamide and 0.02 μM PGE_1, as indicated (final vol. 1.0 ml). All samples contained 0.2% (v/v) dimethyl sulfoxide. Incubations were terminated by addition of trichloroacetic acid (final concentration 5% (w/v)). [^{3}H]cGMP and [^{3}H]cAMP were isolated and each was expressed as a percentage of the corresponding [^{3}H]nucleoside triphosphates (see 2.2.10.). Values are means ± S.E.M. from 3 identical incubation mixtures. Significant increases in cyclic [^{3}H]nucleotides are shown: *, $p < 0.01$.

Additions	[^{3}H]cAMP (% of [^{3}H]ATP)	[^{3}H]cGMP (% of [^{3}H]GTP)
None	0.011 ± 0.000	0.014 ± 0.000
SNP	0.022 ± 0.002*	0.166 ± 0.007*
Cil	0.025 ± 0.000*	0.015 ± 0.002
SNP + cil	0.026 ± 0.001*	0.208 ± 0.004*
PGE_1	0.028 ± 0.001*	0.014 ± 0.050
PGE_1 + SNP	0.241 ± 0.005*	0.153 ± 0.007*
PGE_1 + cil	0.391 ± 0.012*	0.016 ± 0.002
PGE_1 + SNP + cil	0.399 ± 0.015*	0.198 ± 0.003*

Table 3.6. Effects of SNP and M&B 22,948 on [^{3}H]cGMP and [^{3}H]cAMP in rabbit platelets

Platelets were labelled with [^{3}H]guanine and [^{3}H]adenine (see Section 2.2.3.). Samples of the suspension (4 x 10^{8} platelets) were incubated for 0.5 min at 37°C with 0-4.0 μM SNP and 10 μM M&B 22,948. All samples contained 0.2% (v/v) dimethyl sulphoxide. Incubations were terminated by addition of trichloroacetic acid. [^{3}H]cGMP and [^{3}H]cAMP were isolated and expressed as percentages of the corresponding [^{3}H]nucleoside triphosphates; values are means $\pm$ S.E.M and means $\pm$ S.E. of the quotient from 3 identical incubation mixtures.

Additions	M&B 22,948 (10 μM)	$[^3H]$cAMP (% of $[^3H]$ATP)	Potentiation of $[^3H]$cAMP formation by M&B 22,948 (%)	$[^3H]$cGMP (% of $[^3H]$GTP)	Potentiation of $[^3H]$cGMP formation by M&B 22,948 (%)
None	-	0.021 ± 0.000	--	0.012 ± 0.002	--
None	+	0.022 ± 0.001	4.8 ± 4.3	0.016 ± 0.003	30.6 ± 26.9
SNP (0.04 μM)	-	0.039 ± 0.001	--	0.061 ± 0.002	--
SNP (0.04 μM)	+	0.053 ± 0.003	47.2 ± 7.2	0.207 ± 0.019	242.0 ± 32.9
SNP (4 μM)	-	0.063 ± 0.001	--	0.433 ± 0.044	--
SNP (4 μM)	+	0.071 ± 0.001	11.4 ± 7.1	0.650 ± 0.088	50.3 ± 25.4
PGE_1 (0.02 μM)	-	0.036 ± 0.002	--	0.011 ± 0.002	--
PGE_1 (0.02 μM)	+	0.041 ± 0.001	16.6 ± 5.2	0.015 ± 0.001	32.4 ± 27.8
PGE_1 (0.04 μM)	-	0.065 ± 0.003	--	0.011 ± 0.001	--
PGE_1 (0.04 μM)	+	0.089 ± 0.003	35.9 ± 7.8	0.017 ± 0.002	54.0 ± 21.6

Table 3.7. Synergistic effects of SNP and PGE_1 on rat platelet aggregation

WKY rats were anaesthetized with diethyl ether and blood collected by cardiac puncture. Platelets were isolated by differential centrifugation and resuspended in Ca^{2+}-containing medium (Section 2.2.1.). Samples of platelet suspension (0.95 ml containing 4 x 10^9 platelets) were incubated with SNP or PGE_1, either singly or in combination, in siliconized aggregometer tubes for 0.5 min at 37°C. Thrombin (0.5 U/ml) was added to the aggregometer tube and the aggregation recorded after a further 1 min. Values are means ± S.E.M. from 3 determinations.

Additions	Inhibition of aggregation (%)
PGE_1 (0.02 μM)	13 ± 3
SNP (1 μM)	8 ± 2
PGE_1 (0.02 μM)+ + SNP (1 μM)	100 ± 0

Table 3.8. Effects of SNP, cilostamide and PGE_1 on cyclic [^{3}H]nucleotide levels in rat platelets

Blood was collected from diethyl ether-anaesthetized WKY rats. Platelets was isolated by differential centrifugation, labelled with [^{3}H]guanosine and [^{3}H]adenine (see Section 2.2.3.) and resuspended in a Ca^{2+}-containing Tyrode's medium. Samples (0.5 ml containing 2 x 10^8 platelets) were incubated at 37°C with the different additions for 0.5 min. Drugs studied were, PGE_1, SNP and cilostamide (Cil). Incubations were terminated by addition of ice-cold trichloroacetic acid (5% w/v final). Following addition of [^{14}C]cAMP and [^{14}C]cGMP, cyclic [^{3}H]nucleotides were isolated by chromatography on alumina and Dowex 50 (see Section 2.2.8.). Values are expressed as percentages of the corresponding [^{3}H]nucleoside triphosphate and represent means ± S.E.M. from three identical incubations. Values for [^{3}H]cAMP, and [^{3}H]cGMP that are different from their corresponding basal values are indicated, $^*p < 0.05$. Values of [^{3}H]cAMP which are significantly different from those obtained with PGE_1 alone or from the maximum values obtained with both PGE_1 and SNP are indicated; $^a p < 0.02$ and $^b p < 0.02$, respectively.

Additions	[^{3}H]cAMP (% of [^{3}H]ATP)	[^{3}H]cGMP (% of [^{3}H]GTP)
None	0.069 ± 0.003	0.013 ± 0.000
PGE_1 (0.02 μM)	0.127 ± 0.008*	0.010 ± 0.002
SNP (1 μM)	0.085 ± 0.001*	0.054 ± 0.002*
SNP (10 μM)	0.095 ± 0.003*	0.154 ± 0.003*
Cil (1 μM)	0.095 ± 0.003*	0.015 ± 0.001
Cil (10 μM)	0.103 ± 0.002*	0.018 ± 0.001*
PGE_1 (0.02 μM) + SNP (1 μM)	0.216 ± 0.004^a	0.054 ± 0.001*
PGE_1 (0.02 μM) + Cil (1 μM)	0.290 ± 0.005^{a,b}	0.015 ± 0.001
PGE_1 (0.02 μM) + SNP (10 μM)	0.224 ± 0.008^a	0.128 ± 0.009*
PGE_1 (0.02 μM) + Cil (10 μM)	0.574 ± 0.014^{a,b}	0.020 ± 0.003

Chapter 4. *Inhibition of vascular smooth muscle function by nitrovasodilators and isoproterenol*

4.1. *Introduction*

4.1.1. *Effects of nitrovasodilators on platelets and VSM*

The nitrovasodilators, SNP and SIN-1, and EDRF inhibit platelet aggregation (Glusa *et al.*, 1974; Nishikawa *et al.*, 1982; Azuma *et al.*, 1986) in addition to their ability to relax vascular smooth muscle (Hashimoto *et al.*, 1971; Furchgott and Zawadzki, 1980). Both of these effects are associated with the activation of soluble guanylyl cyclase and increases in intracellular cGMP (Murad, 1986; Lincoln, 1989). In contrast, vasodilators such as isoproterenol and PGI_2 exert their effects on vascular smooth muscle by stimulating adenylyl cyclase and increasing intracellular cAMP (Hardman, 1984; Murray, 1990).

4.1.2. *Interactions between activators of adenylyl and guanylyl cyclases*

Several reports have documented synergistic inhibitory effects on platelet aggregation of PGI_2 or its analogues in combination with nitrovasodilators (Levin *et al.*, 1982; De Caterina *et al.*, 1988; Willis *et al.*, 1989; Maurice and Haslam, 1990) or EDRF (Radomski *et al.*, 1987; Macdonald *et al.*, 1988). Whether such

interactions occur in vascular smooth muscle is less clear. However, given that activators of both adenylyl cyclase (PGI_2) and guanylyl cyclase (EDRF) are formed by the vascular endothelium, and that there is evidence suggesting that the release of these compounds may be coupled (de Nucci *et al.*, 1988), such an interaction is plausible and could have marked effects on vessel tone. Evidence for synergism between EDRF and PGI_2 has been obtained in experiments with pig coronary arteries (Shimokawa *et al.*, 1988), whereas comparable synergistic effects were not observed in a study of the effects of SNP and iloprost on rabbit vascular smooth muscle (Lidbury *et al.*, 1989).

4.1.3. *Elements necessary for synergism between activators of adenylyl and guanylyl cyclases are present in some vascular smooth muscle*

The molecular basis of the synergistic inhibition of platelet function caused by the simultaneous actions of activators of guanylyl and adenylyl cyclases was described in Chapter 2. The data presented in that Chapter showed that the synergism resulted from the ability of cGMP to enhance the accumulation of cAMP in platelets, and that this was due to the inhibition of a specific cAMP-PDE by cGMP. This PDE, the cGI-PDE, has been most fully characterized in platelets (Grant and Colman, 1984; MacPhee *et al.*, 1986) and cardiac muscle (Harrison *et al.*, 1986). More recently, a cAMP-PDE with very similar kinetic parameters and sensitivity to inhibitors has been shown to be present

in VSM from rats (Schoeffter *et al.*, 1987; Souness *et al.*, 1989; Lindgren *et al.*, 1990), guinea pigs (Silver *et al.*, 1988), and rabbits (Ahn *et al.*, 1989). Since most VSMC contain soluble guanylyl cyclase, as well as receptor-operated adenylyl cyclase, simultaneous incubation of these tissues with activators of these two signal transduction pathways should cause significant increases in both cGMP and cAMP. Thus, whether the synergism studied in platelets will occur in VSMC must ultimately depend on the presence of the cGI-PDE. Moreover, the effect will be most marked in those tissues where this isozyme plays a major role in the hydrolysis of cAMP.

4.1.4. *Objectives*

In an attempt to determine if an interaction between activators of guanylyl and adenylyl cyclases similar to that observed in platelets occurred in VSMC, the effects of these types of compounds on the vascular contractility of de-endothelialized rat aorta were studied. The relaxation of precontracted rings caused by these agents, as well as their ability to inhibit the contraction of rings which were not precontracted, were studied. The nitrovasodilators used to activate soluble guanylyl cyclase in these experiments were SNP and SIN-1. Isoproterenol was used to activate adenylyl cyclase, since it was known that analogues of PGI_2, such as PGE_1, did not relax rat aorta (Eglen and Whiting, 1988) and that the adenosine-induced relaxation of rat aortic VSM seemed to be, at least in part, endothelium-dependent

(Moritoki *et al.*, 1990). The interactions between the nitrovasodilators and isoproterenol were compared to those between isoproterenol and two selective low K_m cAMP-PDE inhibitors (cilostamide and Ro 20-1724). The cAMP and cGMP levels present in the VSM following treatment with these agents were determined to establish the relative roles of the two cyclic nucleotides in the inhibition of rat function.

4.2. *Results*

4.2.1. Relaxation of pre-contracted rat aortic smooth muscle by vasodilators

Phenylephrine (10 nM - 1 μM) caused a concentration-dependent contraction of de-endothelialized rat aortic rings. Although the maximal tension achieved with different rings was highly variable, a maximum contraction was obtained with 1 μM phenylephrine. The concentration of phenylephrine used in these experiments, 100 nM, caused on average 80% of the maximal contraction. A sub-maximal level of contraction was necessary to ensure that small effects were not misinterpreted or missed. Isoproterenol (30 nM - 2 μM) caused concentration-dependent relaxation of de-endothelialized rat aortic rings precontracted by 100 nM phenylephrine. The IC_{50} of isoproterenol derived from cumulative concentration-response curves (Section 2.2.16.) was 211 ± 21 nM (mean ± S.E.M., 13 determinations,

each based on 3 aortic rings). However, the maximum relaxation varied in different rings, ranging from 30% to 80% of the phenylephrine-induced contraction. The relaxant effect of isoproterenol was freely reversible, as demonstrated by the addition of 1 μM propranolol and by the reproducibility in the same aortic ring of successive contractions induced by phenylephrine, following removal of both phenylephrine and isoproterenol by washing.

Although incubation with isoproterenol caused concentration-dependent relaxation of pre-contracted rat aortic rings, the ability of isoproterenol to cause relaxation was greater in the presence of a low concentration of SNP (Fig. 4.1.). The concentration of SNP used (0.25 nM) was selected because it caused relatively small (< 40%) relaxations when used alone. The size of the effect of SNP alone was important, because only when the effects of two agents are small, is it possible to study synergistic interactions. In order to reduce the errors due to the inherent variability of responses of aortic rings to vasodilators, the effects of these agent, alone or in combination, were tested on the same aortic rings. The potentiation of the action of isoproterenol by SNP was demonstrated by a leftward and upward shift of the isoproterenol concentration-response curves relative to the theoretical additive curves (Fig. 4.1.). This shift in the concentration-response curve is indicative of synergism (Pöch and Holzmann, 1980). SNP caused a 3-fold reduction in the IC_{50} value for isoproterenol, which was statistically signficant ($p < 0.05$) (Table

4.1.). Similarly, a concentration of another nitrovasodilator, SIN-1, which alone relaxed the pre-contracted aorta by less than 40%, also potentiated the relaxation caused by isoproterenol (Fig. 4.2.). Again, 30 nM SIN-1 caused a 3-fold reduction in the IC_{50} for isoproterenol (Table 4.1.).

In the presence of 0.25 nM SNP, which on average caused only 16 ± 6% relaxation alone, the maximum relaxation observed with isoproterenol increased from 55 ± 13% to 84 ± 12% (means ± S.E.M. from 3 experiments). However, both under these conditions and with a lower isoproterenol concentration (96 nM, Fig. 4.3.), the variation in the effects of isoproterenol and SNP were such that these effects were not significantly different from the additive effects of both agents (i.e. supra-additive) ($p < 0.4$). Similar results were obtained with SIN-1 (Fig. 4.3.). Although preincubation of the rat aortic smooth muscle with 30 nM SIN-1 caused a significant reduction in the IC_{50} value for isoproterenol, the relaxations caused by these compounds were not clearly supra-additive (Fig. 4.3.).

As a positive control for the relatively modest effects that the nitrovasodilators had on the isoproterenol-induced relaxation, the potentiation of the relaxant effect of isoproterenol by two cAMP PDE inhibitors, cilostamide and Ro 20-1724, was studied. Both these cAMP PDE inhibitors are known to inhibit cAMP breakdown in rat aortic smooth muscle (Schoeffter *et al.*, 1987), and hence were expected to potentiate the effects of isoproterenol. The effects of these cAMP PDE

inhibitors on the isoproterenol-induced relaxation were virtually identical to those obtained with the nitrovasodilators. Thus, concentrations of these compounds selected to have relaxant effects similar to those obtained with 0.25 nM SNP or 30 nM SIN-1 alone, shifted the isoproterenol concentration-response curves to the left of the theoretical additive curves and increased the maximum relaxation obtained with isoproterenol (Figs. 4.4. and 4.5.). In these studies, cilostamide appeared to be slightly more effective than Ro 20-1724. The 2 to 4-fold shifts in the IC_{50} values for isoproterenol caused by these cAMP PDE inhibitors were statistically significant and similar to those seen with SNP or SIN-1 (Table 4.1.). In 3 experiments, similar to that shown in Fig. 4.4., 20 nM cilostamide increased the maximum relaxation achieved with isoproterenol from an average of 61 ± 10% to 92 ± 4%, although alone it relaxed aortic smooth muscle by only 19 ± 5%. Although this effect of cilostamide did not reach statistical significance ($p < 0.4$), a significant supra-additive effect was observed with cilostamide (but not Ro 20-1724) in the presence of 96 nM isoproterenol (Fig. 4.3.).

Although the potentiating effects of the nitrovasodilators or of the cAMP PDE inhibitors on the isoproterenol-induced relaxations were always significant with respect to changes in the IC_{50} values for isoproterenol, and supra-additive effects of low concentrations of the compounds were sometimes seen, the synergism was not as strong as that observed when the effects of nitrovasodilators and activators of adenylyl cyclase on platelet aggregation were studied (Levin *et*

al., 1982; De Caterina *et al.*, 1988; Willis *et al.*, 1989; Maurice and Haslam, 1990). To address this possible discrepancy, experiments were carried out on the inhibition of aortic smooth muscle contraction, which is probably more closely comparable to inhibition of platelet aggregation than smooth muscle relaxation.

4.2.2. *Effects of vasodilators on the contraction of rat aortic smooth muscle*

Incubation of de-endothelialized rat aortic rings with isoproterenol for 30 s caused a concentration-dependent inhibition of contraction induced by the subsequent addition of 100 nM phenylephrine. This action of isoproterenol required slightly higher concentrations of the compound than relaxation of pre-contracted aortic smooth muscle. An IC_{50} value for isoproterenol of 349 $\pm$ 26 nM was obtained (mean $\pm$ S.E.M., 15 determinations each based on 3 or 4 aortic rings). Under the conditions of our experiments, 10 to 50-fold higher concentrations of nitrovasodilators and of inhibitors of cAMP PDE were required to induce a weak (< 30%) inhibition of contraction than to induce a similar extent of relaxation.

Simultaneous addition of such a concentration of SNP (5 nM) with isoproterenol caused marked inhibition of the phenylephrine-induced contractions (Fig. 4.6.). This effect of SNP on the isoproterenol-induced inhibition of contraction resulted in a leftward

shift in the isoproterenol concentration-response curves relative to the theoretical additive curves (Fig. 4.7.) and a 9-fold reduction in the IC_{50} value for isoproterenol (Table 4.2.). Thus, under these conditions, SNP caused a potentiation of the action of isoproterenol that was about 3-fold the effect observed on relaxation (compare Tables 4.1. and 4.2.). Addition of SIN-1 with isoproterenol also caused synergistic inhibition of phenylephrine-induced contraction (Fig. 4.8.). Thus, 1 μM SIN-1 caused a 9.5-fold reduction in the IC_{50} value for isoproterenol (Table 4.2.). Since the maximum inhibition of contraction obtained with isoproterenol was close to 100%, no upward shift in the concentration-response curves was possible. However, clear supra-additive effects of either SNP or SIN-1 were observed in the presence of 100 nM isoproterenol (Fig. 4.9.).

Both cAMP PDE inhibitors studied (cilostamide and Ro 20-1724) interacted synergistically with isoproterenol to inhibit phenylephrine-induced contractions (Fig. 4.10. and Fig. 4.11.). A concentration of cilostamide (1 μM), which alone had effects similar to those caused by 5 nM SNP or 1 μM SIN-1, shifted the isoproterenol concentration-response curve to essentially the same extent as did either of these nitrovasodilators (Table 4.2.). Ro 20-1724 was only slightly less effective than cilostamide or the nitrovasodilators (Table 4.2.). Supra-additive effects of the above concentrations of these inhibitors of cAMP PDE and 100 nM isoproterenol on inhibition of contraction were also seen (Fig. 4.9.).

The observation that the nitrovasodilators and cAMP-PDE inhibitors studied in this thesis potentiated the inhibition of aortic smooth muscle contraction by isoproterenol to essentially the same extent, and that these compounds also had equal, though weaker, effects on the relaxant action of isoproterenol, suggests that these two different groups of compounds may ultimately act through a common inhibitory mechanism.

4.2.3. *Effects of SNP and isoproterenol on cyclic nucleotide levels in rat aortic smooth muscle*

Concentrations of isoproterenol that inhibited phenylephrine-induced contraction of rat aortic smooth muscle (see Section 4.2.2.) also increased cAMP levels in this tissue (Fig. 4.12a). The basal value of cAMP (0.58 ± 0.04 pmol/mg of protein; mean ± S.E.M.) was increased by 46 ± 29%, 68 ± 26%, 196 ± 55% and 263 ± 73% after 2.5 min incubations with 100 nM, 500 nM, 2 μM and 10 μM isoproterenol, respectively (means ± S.E.M. from 8 determinations). With the exception of 100 nM isoproterenol, these increases in cAMP were statistically significant ($p < 0.02$). Isoproterenol did not affect cGMP levels (Fig. 4.12b). Also, the concentration-dependent increases in aortic smooth muscle cGMP caused by SNP were unaffected by isoproterenol (Fig. 4.12b). The basal value for cGMP (0.17 ± 0.05 pmol/mg of protein; mean ± S.E.M.) was increased by 126 ± 4[illegible]% and 358 ± 132% after 2.5 min incubations with 5 nM and 50 nM SNP, respectively

(means ± S.E.M. from 6-8 determinations). Both of these increases were significant ($p < 0.05$). Although 5 nM SNP did not increase cAMP significantly in the present study, incubation of rat aortic rings with 50 nM SNP, a concentration sufficient to inhibit contraction in response to 100 nM phenylephrine completely in these experiments, did cause a significant increase cAMP (Table 4.3.). This increase in cAMP was similar to that seen with 500 nM isoproterenol under identical experimental conditions (Table 4.3.).

Simultaneous incubation of rat aortic smooth muscle with SNP and low concentrations of isoproterenol greatly increased the accumulation of cAMP caused by the latter (Fig. 4.12a). Thus, on average, 5 nM SNP enhanced the *increases* in cAMP observed in the presence of 100 nM and 500 nM isoproterenol by 4.8-fold and 4.3-fold, respectively. This additional cAMP accumulation was highly significant ($p < 0.001$; Fig. 4.12a). With higher isoproterenol concentrations (2 and 10 μM), 5 nM SNP caused similar increases in the amount of cAMP found in the tissue, though relative to the effect of isoproterenol alone, these increases were smaller (1.8-fold and 1.9-fold, respectively). Substitution of 50 nM SNP for 5 nM caused a somewhat larger increase in the amount of cAMP accumulating during simultaneous incubation with 500 nM isoproterenol (Table 4.3.). Although 50 nM SNP itself increased cAMP, the effects of the two compounds in combination were clearly supra-additive ($p < 0.001$). Isoproterenol (100 nM or 500 nM) did not affect the increases in cGMP caused by 5 or 50 nM SNP (Fig. 4.12b and Table 4.3.).

4.3. *Discussion*

4.3.1. *Synergistic interactions between isoproterenol and nitrovasodilators or cAMP PDE inhibitors*

In experiments with rat aortic rings from which the endothelium had been removed, the nitrovasodilators, SNP and SIN-1, potentiated the ability of isoproterenol to relax pre-contracted aortic smooth muscle and, when added with isoproterenol before the contractile stimulus, these agents exerted potent synergistic inhibitory effects on contraction. In contrast, a study of the interactions between SNP and iloprost in VSMC failed to detect synergism (Lidbury *et al.*, 1989). Although use of rabbit mesenteric and coeliac arteries in the latter study, rather than rat aorta, could account for the different results obtained, other considerations may be more important. In our experiments, detection of the synergistic effects of nitrovasodilators and isoproterenol on relaxation depended on comparison of results obtained in the same aortic rings incubated with and without test compounds. Moreover, potentiation of the effects of isoproterenol by either the nitrovasodilators or the cAMP PDE inhibitors was always much more marked when inhibition of contraction, rather than relaxation, was studied. The reasons for the latter difference are not clear, but both methodological and physiological factors could be important.

First, prolonged exposure of pre-contracted smooth muscle to nitrovasodilators or cAMP PDE inhibitors could have inhibitory, as well as stimulatory, effects on the ability of the tissue to respond to isoproterenol. Thus, pretreatment of other rat tissues with cAMP analogues has been shown to increase cAMP PDE activity and to reduce the effects of agonists that stimulate adenylyl cyclase (Gettys *et al.*, 1987). In addition, the platelet cGI-PDE appears to be activated by cAMP-dependent phosphorylation (MacPhee *et al.*, 1988; Grant *et al.*, 1988). The continued presence of phenylephrine could also have adversely affected the actions of the other compounds studied, for example through the activation of protein kinase C.

Second, there may be critical differences in the state of the VSMC before and after induction of contraction that affect the responses to the vasodilator drugs studied. Since increases in intracellular Ca^{2+} concentration and the phosphorylation of MLC are important in the contraction of vascular smooth muscle (Kamm and Stull, 1985; Murphy, 1989), compounds that block these processes will inhibit contraction. On the other hand, relaxation of smooth muscle, which involves reversal of a latch state, is less dependent on the inhibition of Ca^{2+} mobilization or on dephosphorylation of myosin (Dillon *et al.*, 1981; Hai and Murphy, 1988). Increases in cGMP, presumably acting through cGMP-PK, reduce the level of myosin phosphorylation in smooth muscle (Rapoport *et al.*, 1983), activate the plasmalemmal Ca^{2+}-ATPase (Rashatwar *et al.*, 1987; Vrolix *et al.*, 1988; Cornwell and

Lincoln, 1989) and inhibit the G-protein-mediated stimulation of phosphoinositide hydrolysis (Hirata *et al.*, 1990). On the other hand, cAMP-PK activity increases the concentration of Ca^{2+}-calmodulin required for activation of MLCK (Adelstein *et al.*, 1978) and the Ca^{2+}-sensitivity of smooth muscle Ca^{2+}-dependent potassium channels (Sadoshima *et al.*, 1988). Thus, in view of the different biochemical mechanisms that may be involved in the inhibition of contraction and relaxation, it is entirely conceivable that activation of one or the other cyclic nucleotide systems, or both together, could have distinct effects. The cyclic nucleotide regulated protein kinases are a site at which an interaction between the two cyclic nucleotides could potentiate their individual effects. Indeed, binding of cAMP to one of the two cyclic nucleotide binding sites of the cGMP-PK has been shown to increase the affinity of the other site for cGMP. In addition, activation of the cGMP-PK by cAMP has been shown to be responsible for mediating some of the effects of this cyclic nucleotide in cultured VSMC (Lincoln *et al.*, 1990).

The marked synergism between the actions of the nitrovasodilators and isoproterenol reported here for the inhibition of rat smooth muscle contraction is analogous to that observed in platelets between nitrovasodilators and activators of platelet adenylyl cyclase (Levin *et al.*, 1982; De Caterina *et al.*, 1988; Willis *et al.*, 1989; Maurice and Haslam, 1990). In platelets, the synergism results from the inhibition by cGMP of cAMP breakdown by a cGI-PDE (Maurice and Haslam,

1990). An enzyme with kinetic characteristics and a sensitivity to inhibitors very similar to that of the platelet enzyme has been shown to be present in rat, guinea pig, and rabbit aorta (Schoeffter *et al.*, 1987; Silver *et al.*, 1988; Souness *et al.*, 1989; Ahn *et al.*, 1989). The possibility therefore exists that the synergism between nitrovasodilators and isoproterenol reported here results from inhibition by cGMP of this same enzyme in rat aortic smooth muscle. Much of the data is consistent with this. First, both SNP and SIN-1 had potentiating effects on the action of isoproterenol that were virtually indistinguishable from those obtained using cilostamide, whether relaxation or inhibition of contraction was studied. The latter compound has been shown to be a selective inhibitor of the cGI-PDE in platelets (Hidaka *et al.*, 1979; MacPhee *et al.*, 1986) and to increase cAMP, but not cGMP levels in rat aortic smooth muscle at concentrations that caused 50% relaxation (Schoeffter *et al.*, 1987). The possibility that the nitrovasodilators do potentiate the effects of isoproterenol by inhibiting the cGI-PDE enzyme is also consistent with evidence that cilostamide appears to decrease relaxation by SNP in rat aortic smooth muscle (Schoeffter *et al.*, 1987). Ro 20-1724, a compound known to inhibit a different low K_m cAMP PDE that is unaffected by cGMP (Section 1.14.4.), was slightly less effective in potentiating the actions of isoproterenol. This observation suggests that cAMP breakdown in vascular smooth muscle depends on the activities of both low K_m cAMP PDEs. Consistent with this, recent reports have shown that inhibition of either of these enzymes with pharmacological

inhibitors brings about relaxation of VSM (Lindgren *et al.*, 1990, Silver *et al.*, 1989). However, the relative importance of the two activities seems to be tissue specific (Silver *et al.*, 1989).

EDRF has been identified as nitric oxide (Palmer *et al.*, 1987) or more probably an *S*-nitrosothiol (Myers *et al.*, 1990) and appears to relax VSMC by the same cGMP-dependent mechanism as do the nitrovasodilators (Murad, 1986; Lincoln, 1989). A synergism similar to that seen with nitrovasodilators should therefore be observed between the effects of EDRF and activators of adenylyl cyclase on VSMC. It is therefore significant that physiological stimuli can cause a coupled release of both EDRF and PGI_2 from the vascular endothelium (De Nucci *et al.*, 1988) and that an interaction has been observed between EDRF in an endothelial superfusate and PGI_2 in causing the relaxation of pig coronary artery (Shimokawa *et al.*, 1988). These results are consistent with the known synergistic effects of EDRF and PGI_2 as inhibitors of platelet aggregation (Radomski *et al.*, 1987; Macdonald *et al.*, 1988).

The present results extend evidence of significant synergistic interactions between nitrovasodilators and activators of adenylyl cyclase in platelets to VSMC and provides indirect evidence that the same mechanism, namely inhibition by cGMP of a low K_m cAMP PDE, may be responsible.

4.3.2. *Potentiation of the isoproterenol-induced increases in rat aortic smooth muscle cAMP by SNP is consistent with effects of these agents on function*

It is generally accepted that nitrovasodilators exert their effects on vascular smooth muscle by activating guanylyl cyclase and increasing cGMP (Murad, 1986; Lincoln, 1989) and that β-adrenergic agonists act by stimulating adenylyl cyclase and increasing cAMP (Levitzki, 1987). These mechanisms have generally been assumed to be parallel and independent. In experiments described in Section 4.2.3., addition of SNP or of isoproterenol alone caused concentration-dependent increases in rat aortic cGMP or cAMP, respectively. These effects were consistent with those reported by others using this tissue (Lincoln, 1983; Schoeffter and Stoclet, 1982). However, when low concentrations of both of these compounds were added simultaneously, 4 to 5-fold greater increases in cAMP were obtained than with isoproterenol alone. In contrast, the increases in cGMP caused by SNP were not affected by isoproterenol. These results indicate that cAMP rather than cGMP is likely to mediate the synergistic inhibition of rat aortic smooth muscle contraction seen when both SNP and isoproterenol are present. Although a synergistically effective concentration of SNP (5 nM) did not appear to affect tissue cAMP when the nitrovasodilator alone was present, a higher concentration of SNP (50 nM) did increase cAMP significantly. Comparison of the latter effect with the increases in cAMP caused by isoproterenol suggest that cAMP could have contributed to the inhibition

of contraction by this concentration of SNP. Most workers have not reported increases in cAMP in VSMC preparations incubated with nitrovasodilators (Diamond and Blisard, 1976; Kukovetz *et al.*, 1979; Axelsson *et al.*, 1982; Rapoport and Murad, 1983; Kukovetz and Holzmann, 1986; Martin *et al.*, 1986; Schoeffter *et al.*, 1987; Ahler *et al.*, 1988; Tanaka *et al.*, 1988). Although the technical reasons discussed in Section 3.3.1. also apply to the measurements of changes in cyclic nucleotides in smooth muscle, in this tissue there are additional complicating factors. Thus, it is much more difficult to obtain homogeneous samples of smooth muscle tissue than it is to obtain homogeneous platelet suspensions. Sample heterogeneity may also result from differences in the responses of cells derived from distinct regions of the artery, or vein, under study. Again, with platelets this is much less important. In many of the studies cited above, very high concentrations of nitrovasodilator were used. Since VSMC could also have cGS-PDE activity (Section 1.14.2.), the large increases in cGMP which occurred may not have had a net inhibitory effect on the hydrolysis of cAMP.

In platelets, in which increases in cAMP can be measured much more accurately, SNP has been shown to causes small increases in cAMP by itself, in addition to potentiating the increases in cAMP seen in the presence of activators of adenylyl cyclase (Chapter 3). This effect of SNP was attributable to inhibition of cGI-PDE in platelets by cGMP (see Chapter 3 and Maurice and Haslam, 1990). The latter enzyme has recently

been shown to be partly responsible for cAMP breakdown in rat aortic smooth muscle (Lindgren *et al.*, 1990) and has been purified from bovine aortic smooth muscle (Rascon *et al.*, 1989). Thus, it is plausible that inhibition of the smooth muscle cGI-PDE by cGMP mediates the synergistic effects of SNP and isoproterenol on cAMP accumulation that have observed in this tissue, as well as the smaller increase in cAMP seen with 50 nM SNP alone.

The results described in this Chapter, suggest that cAMP formation in VSMC could play a previously unsuspected role in the actions of nitrovasodilators and EDRF, which under physiological conditions are likely to function in the presence of endogenous activators of adenylyl cyclase. In this context, it may also be relevant that both cGMP and cAMP levels are lower in de-endothelialized than intact arteries (Rapoport and Murad, 1983). Thus, the results may offer a partial explanation of observations showing that vasodilators, such as isoproterenol, adenosine and PGI_2, that potentially exert direct effects on vascular smooth muscle through cAMP formation, are much more effective when the endothelium, and therefore EDRF, is present (Rubanyi and Vanhoutte, 1985; Shimokawa *et al.*, 1988). Finally, a role for cAMP in the actions of nitrovasodilators may help to explain some of the quantitative discrepancies between cGMP formation and relaxation in smooth muscle noted in a recent review (Nakatsu and Diamond, 1989). For example, both Molina *et al.* (1987) and Keith *et al.* (1982) reported that the relaxation of rat aortic smooth muscle caused by

nitrovasodilators (nitroglycerin or SNP) were not correlated with the increases in cGMP caused by these agents. Small increases in cAMP caused by the nitrovasodilators may account for the discrepancies.

4.4. *Summary*

4.4.1. Isoproterenol caused a concentration-dependent relaxation of de-endothelialized rat aortic rings pre-contracted with 100 nM phenylephrine and, when added before the contractile agent, a similar concentration-dependent inhibition of contraction. The concentrations of isoproterenol required to inhibit phenylephrine-induced contractions were slightly higher than those required to relax rings precontracted to phenylephrine (IC_{50} values 211 $\pm$ 11 nM and 349 $\pm$ 26 nM, respectively).

4.4.2. Nitrovasodilators (SNP or SIN-1) and cAMP phosphodiesterase inhibitors (cilostamide or Ro 20-1724) relaxed de-endothelialized rat aortic rings pre-contracted with phenylephrine. Much higher concentrations of these compounds was required to inhibit contraction than to induce a similar degree of ralaxation. The differences involved ranged from 20 to 50-fold.

4.4.3. Treatment of pre-contracted rat aortic rings with low concentrations of either of the nitrovasodilators caused a significant (2 to 4-fold) decrease in the IC_{50} value for the isoproterenol-induced relaxations.

4.4.4. Simultaneous additions of isoproterenol and threshold concentrations of either of the two nitrovasodilators caused synergistic inhibitions of contraction, with a 9 to 10-fold decrease in the IC_{50} values for isoproterenol. Again, when the cAMP phosphodiesterase inhibitors replaced the nitrovasodilators, they too potentiated the effects of isoproterenol.

4.4.5. Thus, synergism between nitrovasodilators and isoproterenol, could be detected in vascular smooth muscle and was particularly marked when inhibition of contraction was studied. The action of the two nitrovasodilators in both systems studied resembled that of inhibitors of cAMP phosphodiesterase.

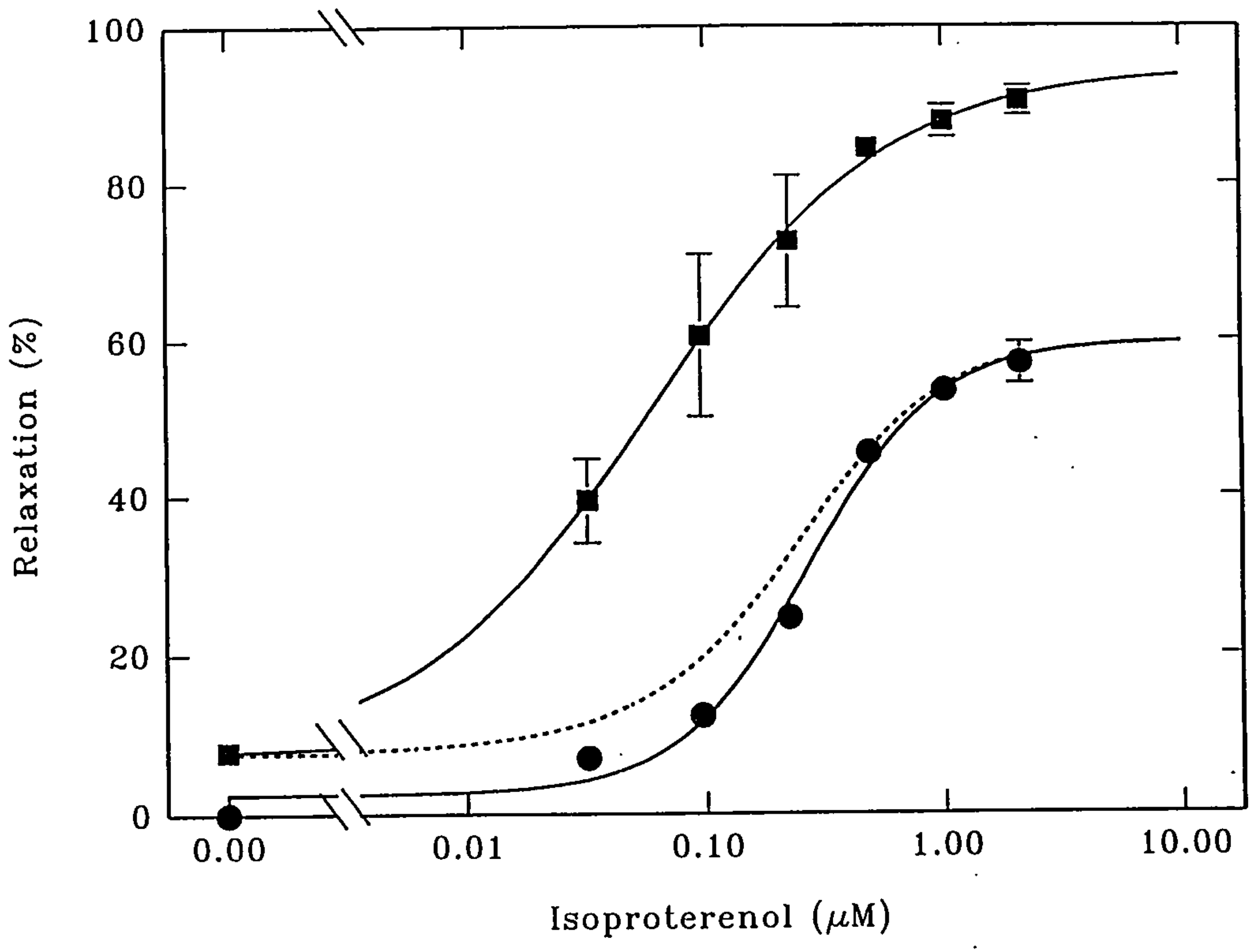
4.4.6. In experiments with concentrations of SNP (e.g. 5 nM) and isoproterenol (e.g. 100 nM) that caused synergistic inhibitions of contraction, SNP not only increased smooth muscle cGMP but also enhanced the increases in smooth muscle cAMP caused by isoproterenol by 4 to 5-fold, whereas the SNP-induced increases in tissue cGMP were unaffected by isoproterenol.

4.4.7. Although low concentrations of SNP (5 nM) alone did not increase smooth muscle cAMP, a higher concentration (50 nM) significantly increased cAMP levels and also caused a supra-additive increase in cAMP with a high isoproterenol concentration (500 nM).

4.4.8. cAMP, rather than cGMP, is likely to mediate the synergistic inhibition of the contraction of rat aortic smooth muscle caused by nitrovasodilators and isoproterenol.

Fig. 4.1. Effect of SNP on isoproterenol-induced relaxation of rat aortic rings pre-contracted by 100 nM phenylephrine

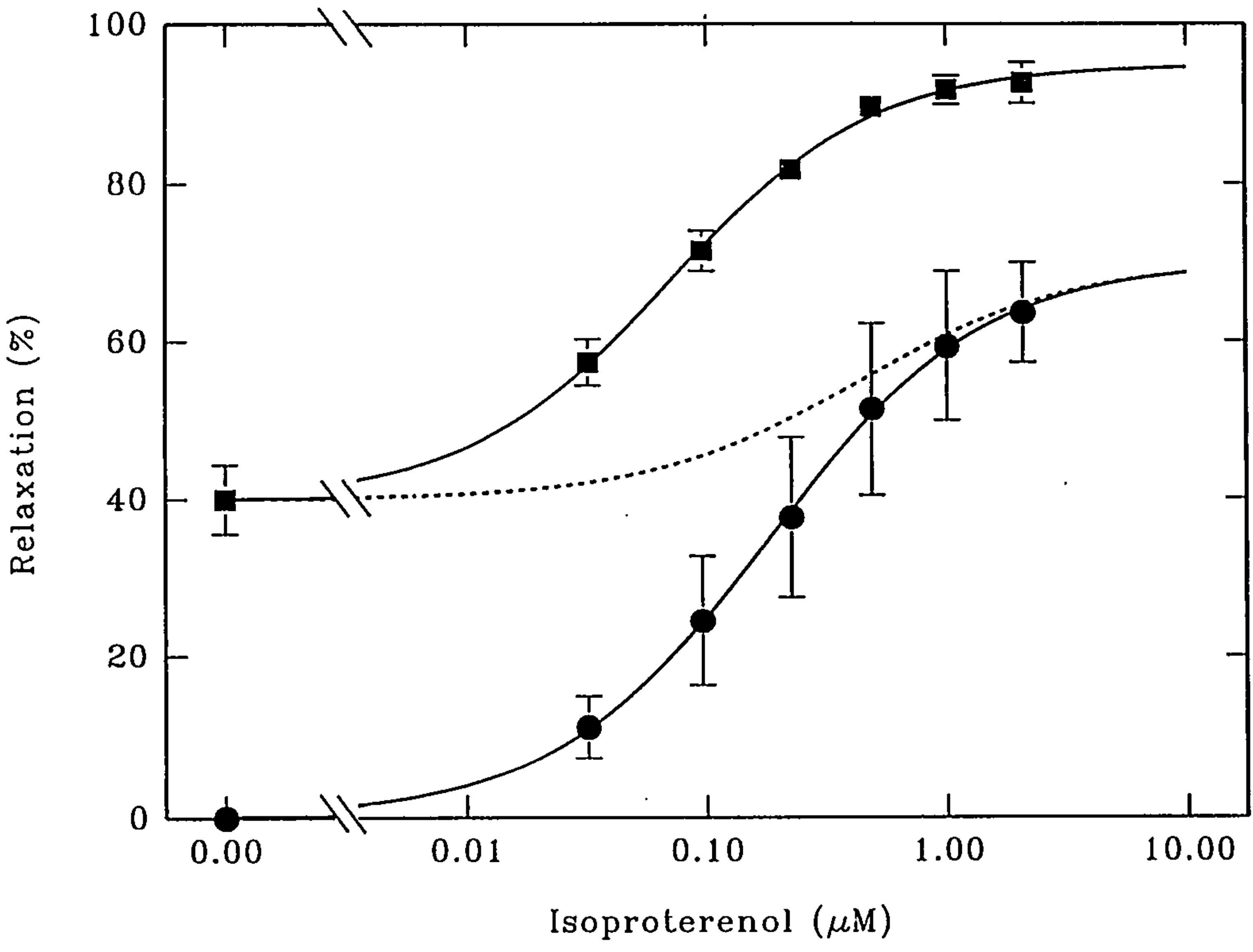
Cumulative concentration-response curves for isoproterenol were obtained in both the absence (●) and presence (■) of 0.25 nM SNP, as described in Section 2.2.16. Values are means ± S.E.M. from 3 aortic rings. The theoretical additive concentration-response curves are also shown (---) (see Section 2.2.18.).



Relaxation (%)
100
80
60
40
20
0
0.00
0.01
0.10
1.00
10.00
Isoproterenol (μM)

Fig. 4.2. Effect of SIN-1 on the isoproterenol-induced relaxation of rat aortic rings pre-contracted by 100 nM phenylephrine

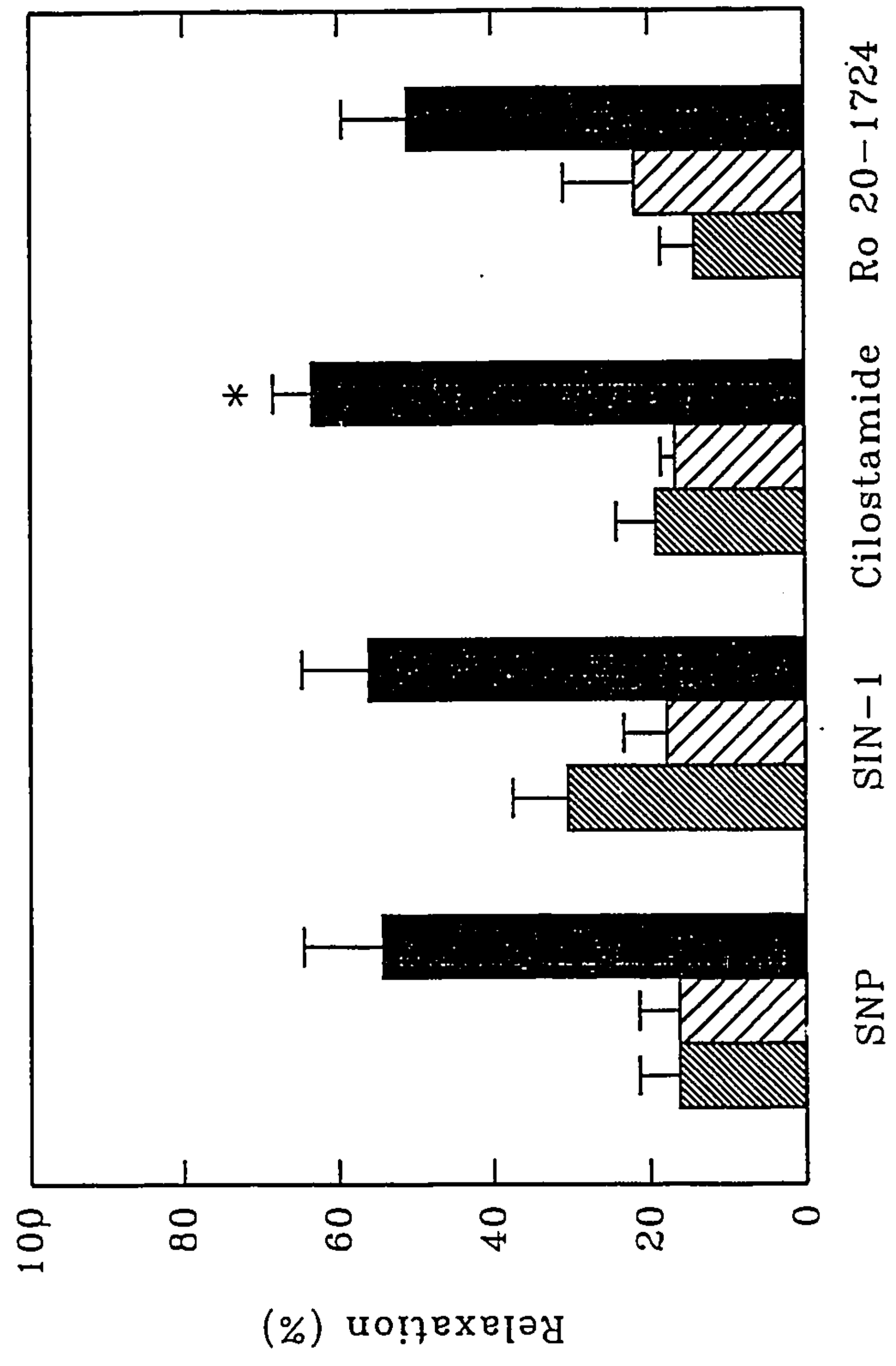
Cumulative concentration-response curves for isoproterenol were obtained in both the presence (■) and the absence (●) of 30 nM SIN-1. Values are means ± S.E.M. from 3 rings. The theoretical additive curve is also shown (--) (see Section 2.2.18.).



Relaxation (%)
100
80
60
40
20
0
0.00
0.01
0.10
1.00
10.00
Isoproterenol (μM)

Fig. 4.3. Effects of nitrovasodilators and of cAMP phosphodiesterase inhibitors on the relaxation by 96 nM isoproterenol of rat aortic rings pre-contracted by 100 nM phenylephrine

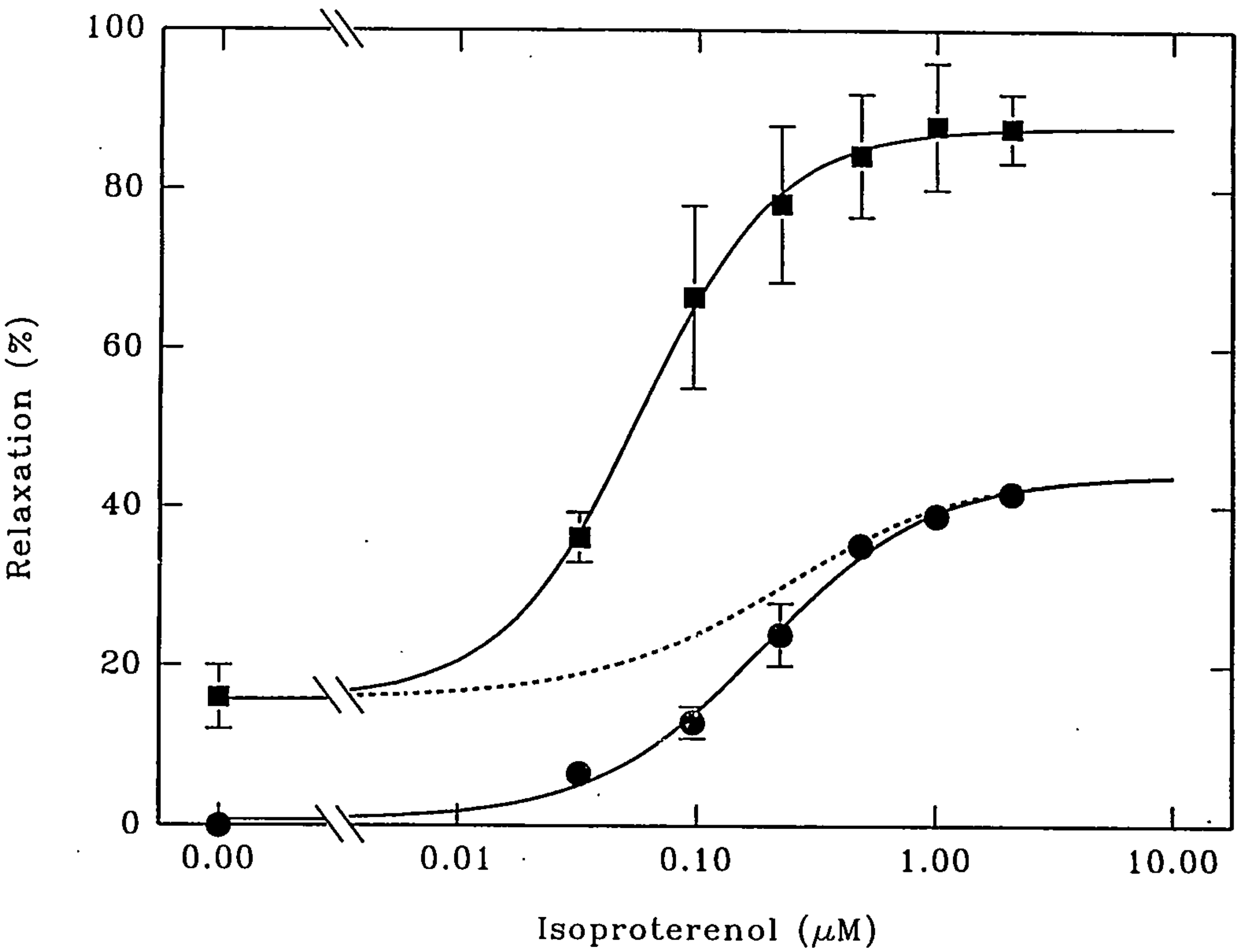
The compounds studied were SNP (0.25 nM), SIN-1 (30 nM), cilostamide (20 nM) and Ro 20-1724 (10 M). Results with 96 nM isoproterenol are from cumulative concentration-response curves similar to those shown in Figs. 4.1 and 4.2.. The first bar in each group shows the percent relaxation with the indicated compound alone ( ), the second, the relaxation of the same aortic rings with isoproterenol alone ( ) and the third, the relaxation induced by the combined action of both compounds ( ). Results are means $\pm$ S.E.M. from 3 experiments, each with 3 or 4 aortic rings. The significant supra-additive effect of cilostamide and isoproterenol is indicated: * $p < 0.05$.



Relaxation (%)
0
20
40
60
80
100
SNP
SIN-1
Cilostamide
Ro 20-1724
*

Fig. 4.4. Effect of cilostamide on isoproterenol-induced relaxation of rat aortic rings pre-contracted by 100 nM phenylephrine

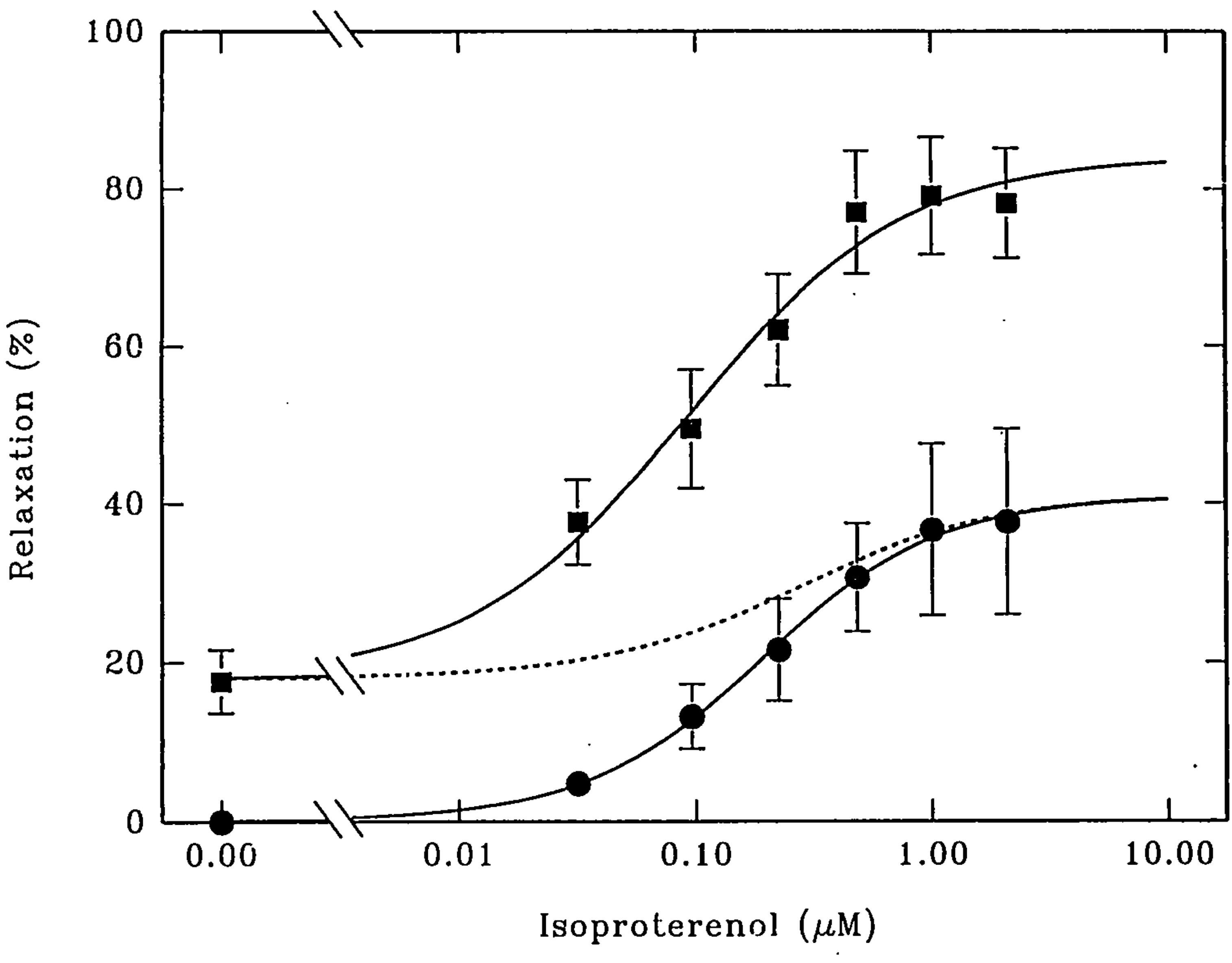
Cumulative concentration-response curves for isoproterenol were obtained in both the presence (■) and absence (●) of 20 nM cilostamide, as described in Section 2.2.16. Values are means ± S.E.M. from 3 aortic rings. The theoretical additive concentration-response curve is also shown (---) (see Section 2.2.18.).



Relaxation (%)
100
80
60
40
20
0
0.00
0.01
0.10
1.00
10.00
Isoproterenol (μM)

Fig. 4.5. Effect of Ro 20-1724 on isoproterenol-induced relaxation of rat aortic rings pre-contracted with 100 nM phenylephrine

Concentration-response curves for isoproterenol were obtained in both the presence (■) and the absence (●) of 10 M Ro 20-1724. Values are means ± S.E.M. from 3 aortic rings. The theoretical additive curve (--) was calculated as described in Section 2.2.18.



Relaxation (%)
0
20
40
60
80
100
0.00
0.01
0.10
1.00
10.00
Isoproterenol (μM)

Fig. 4.6. Effects of isoproterenol and SNP on the contraction of rat aortic smooth muscle by phenylephrine

Shown are representative recordings obtained from one aortic ring incubated for 30 s with (1) no additions, (2) 100 nM isoproterenol (I), (3) 5 nM SNP (S), or (4) 100 nM isoproterenol and 5 nM SNP (I+S) before addition of 100 nM phenylephrine (P).

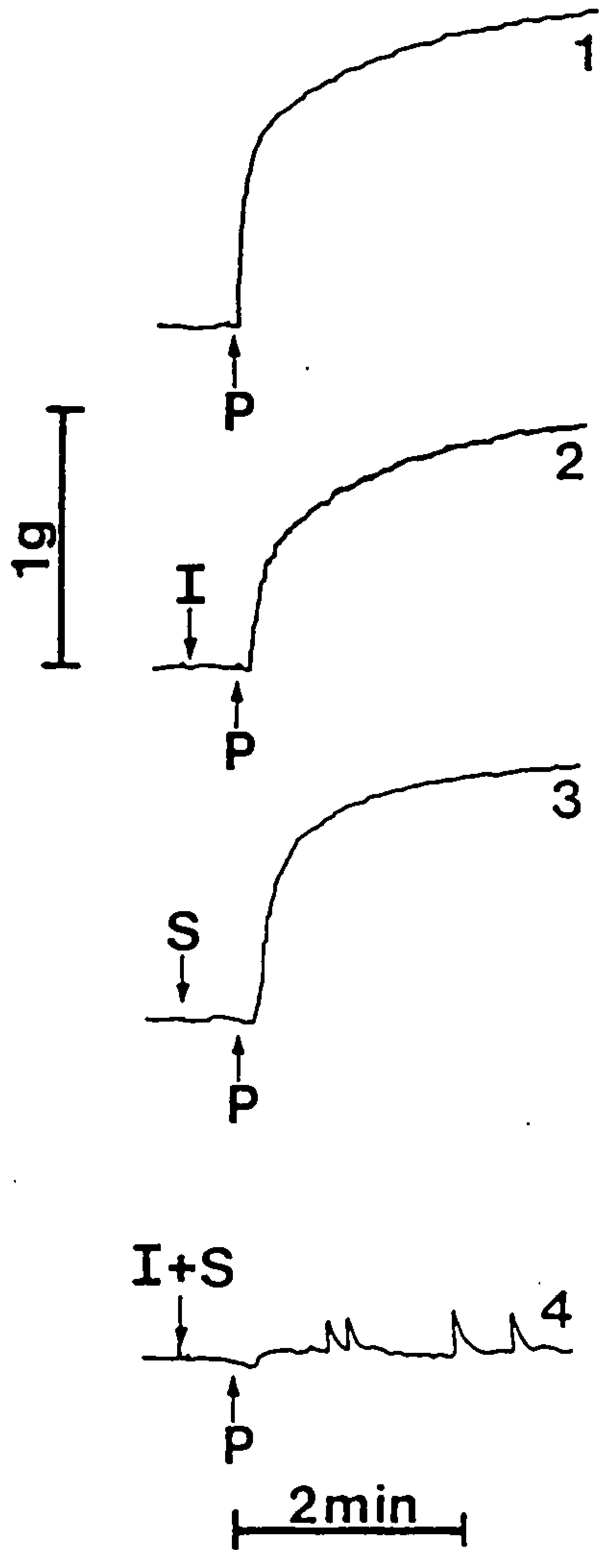

1
P
1g
2
I
P
3
S
P
I+S
4
P
2min

Fig. 4.7. Effect of SNP on the ability of isoproterenol to inhibit phenylephrine-induced contractions of rat aortic rings

The indicated concentrations of isoproterenol were added without (●) or with (■) 5 nM SNP. After 30 s, rings were contracted with 100 nM phenylephrine. Contractions were measured after a further 2 min, and were expressed as percentages of controls with phenylephrine alone. Values are means ± S.E.M. from 4 rings. The theoretical additive concentration-response curve is also shown (---) (see Section 2.2.18.).

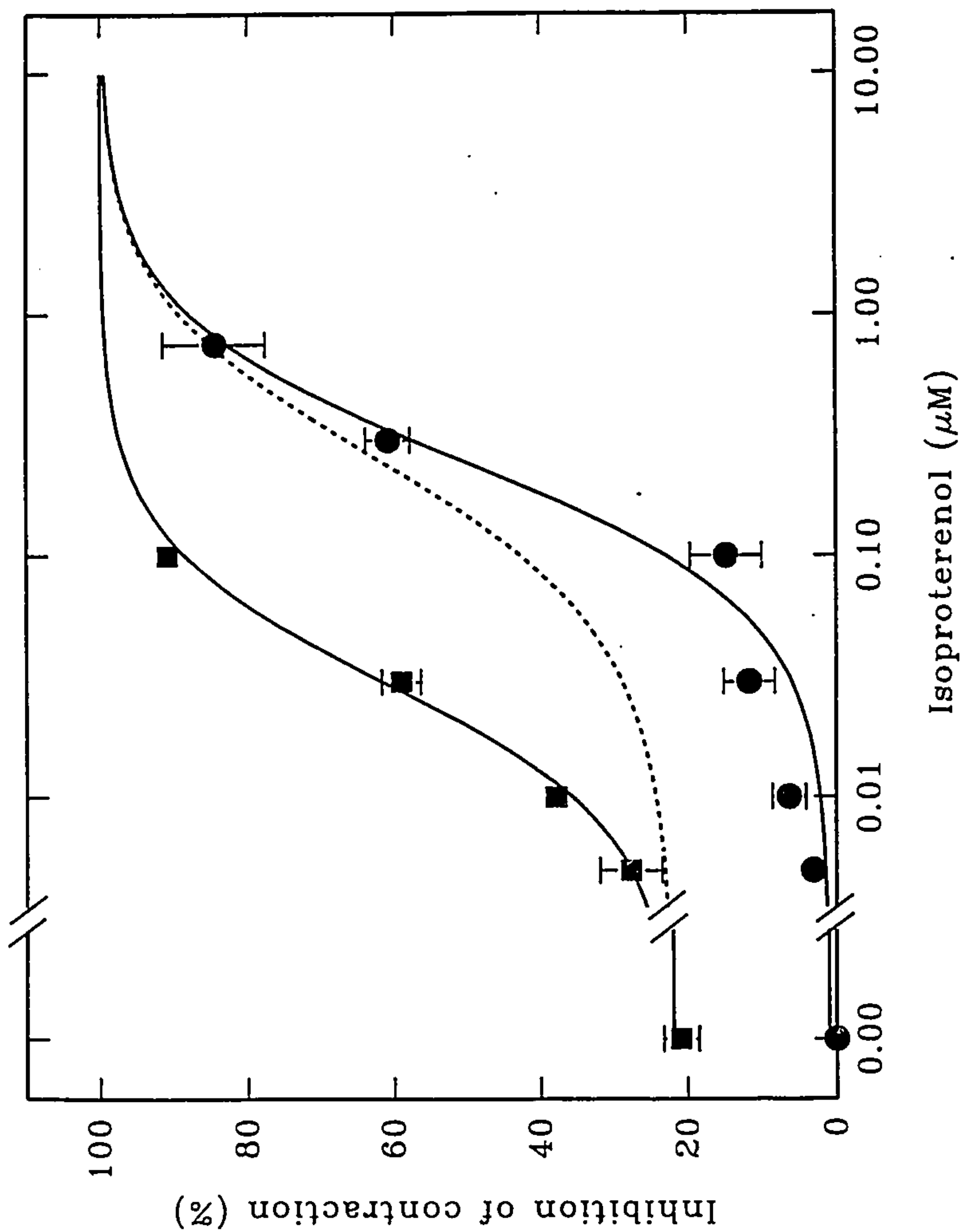

Inhibition of contraction (%)
0
20
40
60
80
100
Isoproterenol (μM)
0.00
0.01
0.10
1.00
10.00

Fig. 4.8. Effect of SIN-1 on the ability of isoproterenol to inhibit phenylephrine-induced contractions of rat aortic rings

The rat aortic smooth rings were incubated for 30s with the indicated concentrations of isoproterenol without (●) or with (■) 1 μM SIN-1 before addition of 100 nM phenylephrine. Contractions were measured after a further 2 min, and expressed as percentages of controls with phenylephrine alone. Values are means ± S.E.M. from 4 rings. The theoretical additive curve (--) is also shown (see Section 2.2.18.).

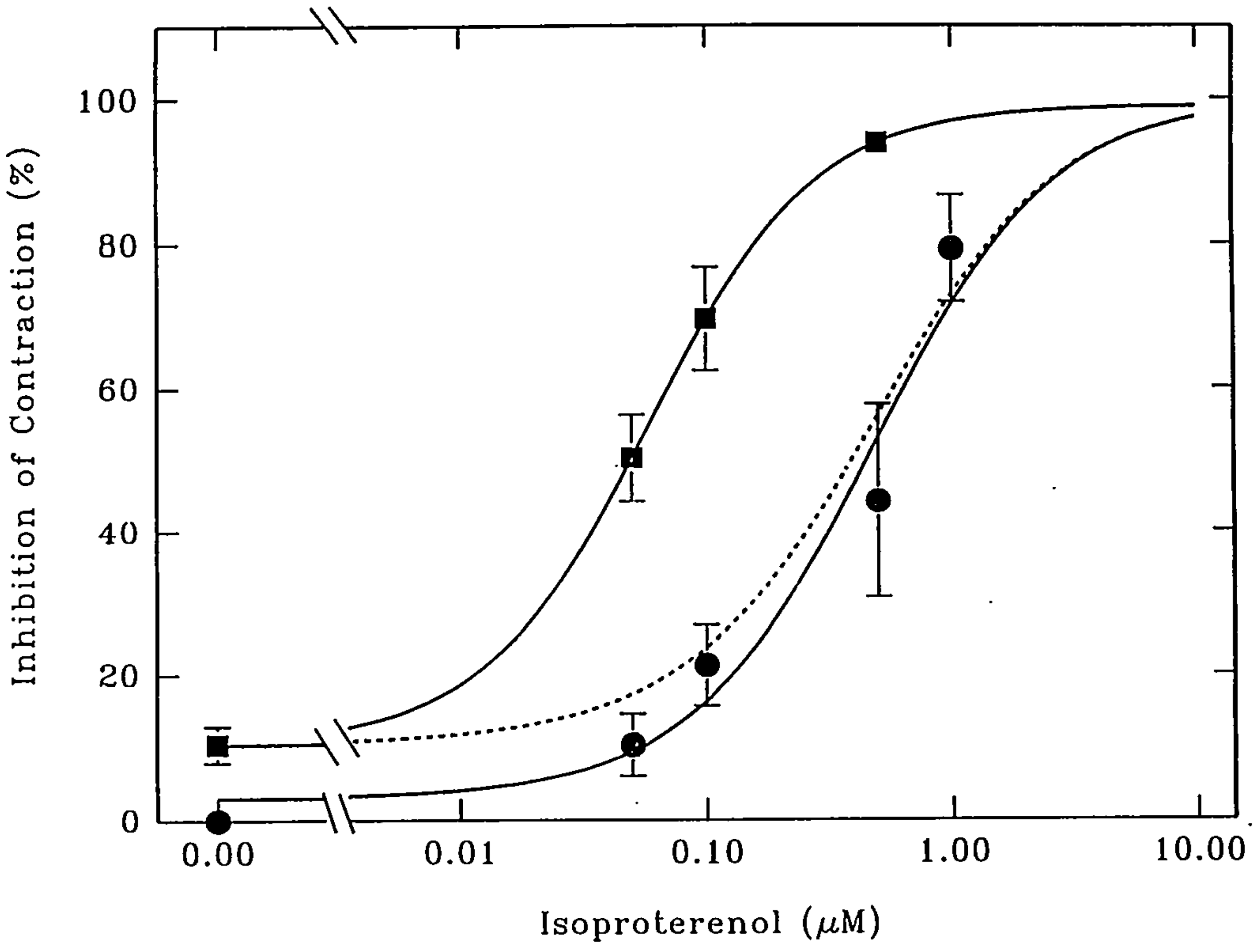

100
80
60
40
20
0
Inhibition of Contraction (%)
0.00
0.01
0.10
1.00
10.00
Isoproterenol (μM)

Fig. 4.9. Effects of nitrovasodilators and of cAMP phosphodiesterase inhibitors on the ability of 100 nM isoproterenol to inhibit the contraction of rat aortic rings by 100 nM phenylephrine

The compounds studied were SNP (5 nM), SIN-1 (1 μM), cilostamide (1 μM) and Ro 20-1724 (100 μM). Rings were incubated for 30 s with isoproterenol without or with one of the above compounds. Phenylephrine was then added and the resulting contraction measured after a further 2 min. The first bar in each group shows the inhibition of contraction by the indicated compound alone (▨), the second, the inhibition of contraction by isoproterenol alone (▧) and the third, the combined action of both compounds (■). Values are means ± S.E.M. from 3 to 6 experiments, each with 3 or 4 aortic rings. Supra-additive effects of the drug combinations studied are indicated: * $p < 0.05$; ** $p < 0.01$.

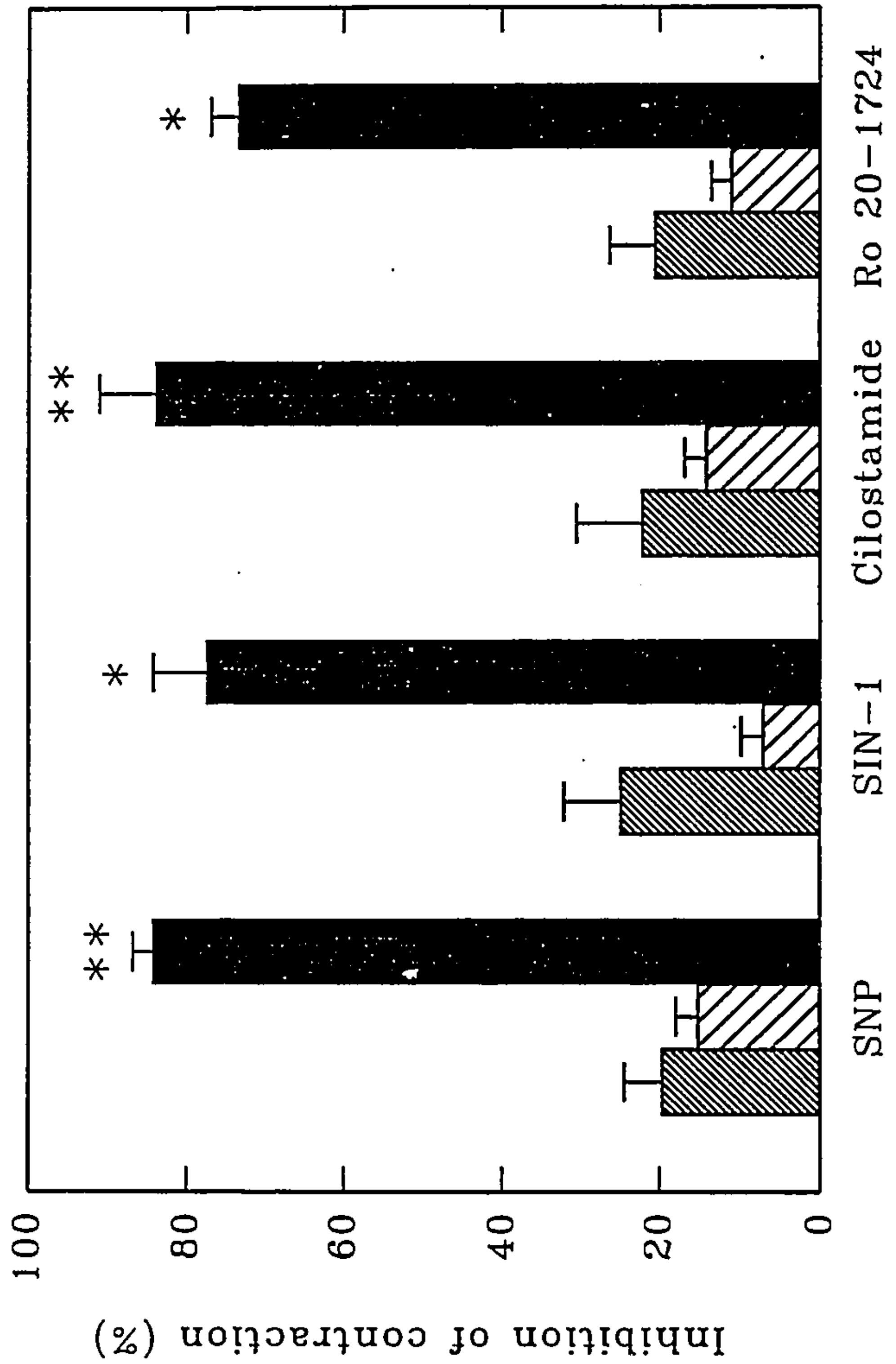

Inhibition of contraction (%)
0
20
40
60
80
100
SNP
SIN-1
Cilostamide
Ro 20-1724
*
**

Fig. 4.10. Effect of cilostamide on the isoproterenol-induced inhibition of the contraction of rat aortic rings by phenylephrine

Rat aortic rings were incubated with isoproterenol in the absence (●) or in the presence of (■) 1 μM cilostamide. After 30 s, rings were contracted by the addition of 100 nM phenylephrine. After a further 2 min the contraction was measured and compared to the contraction of these rings by phenylephrine alone. Values are means ± S.E.M. from 4 rings. The theoretical additive concentration-response curve is also shown (--) (see Section 2.2.18.).

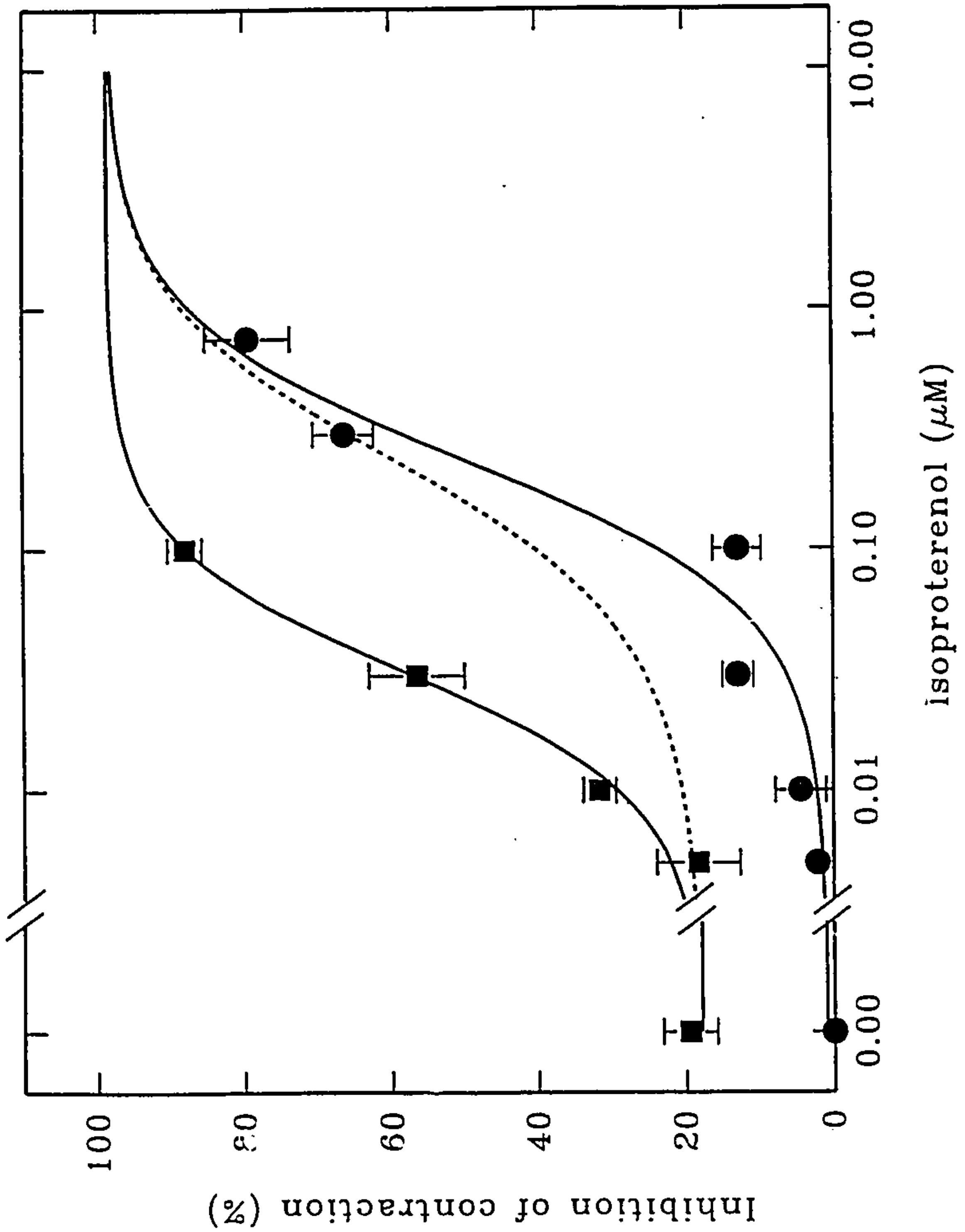

Inhibition of contraction (%)
0
20
40
60
80
100
Isoproterenol (μM)
0.00
0.01
0.10
1.00
10.00

Fig. 4.11. Effects of Ro 20-1724 on the ability of isoproterenol to inhibit phenylephrine-induced contractions of rat aortic rings

The indicated concentrations of isoproterenol were added with (■) or without (●) 100 μM Ro 20-1724. After 30 s, rings were contracted with 100 nM phenylephrine. Contractions were measured after a further 2 min, and were expressed as percentages of controls with phenylephrine alone. Values are means ± S.E.M. from 4 different rings. The theoretical additive concentration-response curve is shown (---) (see Section 2.2.18.).

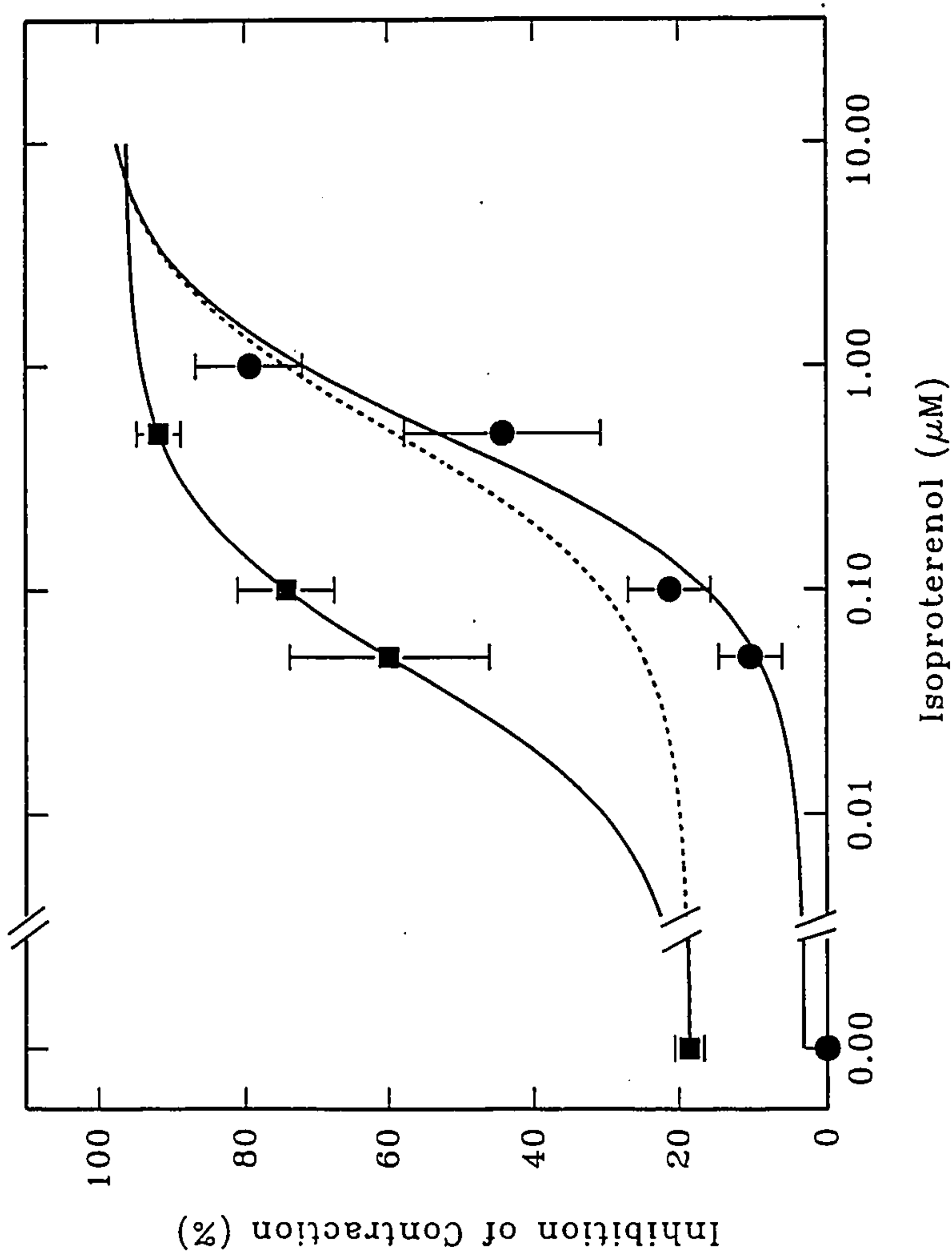

Inhibition of Contraction (%)
0
20
40
60
80
100
Isoproterenol (μM)
0.00
0.01
0.10
1.00
10.00

Fig. 4.12. Effects of SNP and isoproterenol on cyclic nucleotide levels in rat aortic smooth muscle

Rat aortic rings were incubated with the indicated concentrations of isoproterenol for 2.5 min in the absence (▨) or presence (■) of 5 nM SNP. Tissue cAMP (a) and cGMP (b) were then determined. Values are means ± S.E.M. from 6-8 separate incubations, each containing 2 aortic rings. Significant effects of SNP relative to those obtained with the indicated isoproterenol concentrations are shown; $^{*}p < 0.005$, $^{**}p < 0.001$.

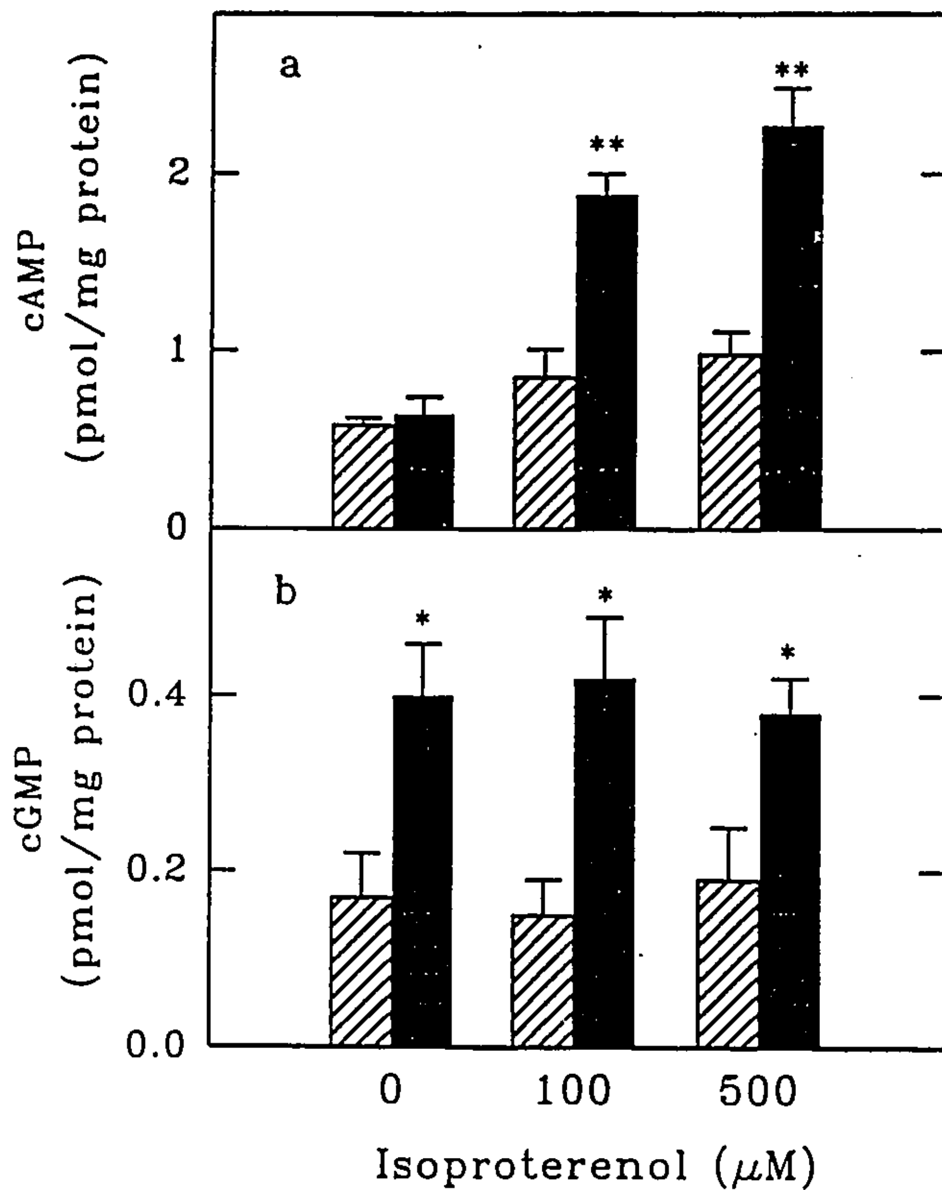

a
b
cAMP
(pmol/mg protein)
cGMP
(pmol/mg protein)
0
1
2
0.0
0.2
0.4
**
**
*
*
*
0
100
500
Isoproterenol (μM)

Table 4.1. Effects of low concentrations of nitrovasodilators and of inhibitors of cAMP phosphodiesterase on the ability of isoproterenol to relax rat aortic rings previously contracted by 100 nM phenylephrine

IC_{50} values for isoproterenol were determined from cumulative concentration-response curves, as described in Section 2.2.18. Each experiment was similar to that shown in Fig. 4.1. and yielded paired values for the IC_{50} of isoproterenol in the absence and presence of the indicated compounds. Mean values ± S.E.M. are given from the numbers of experiments indicated; the significance of changes in IC_{50} for isoproterenol were evaluated by two-sided paired *t* tests after logarithimic transformation of the results; $^{*}p < 0.05$, $^{**}p < 0.01$. The ratio of IC_{50} values for isoproterenol obtained in the absence and presence of each compound studied indicates the extent of synergism.

Compound studied	No. of expts.	IC_{50} of isoproterenol (nM)		Ratio of IC_{50} values
		Without compound	With compound	
SNP (0.25 nM)	4	181 ± 42	69 ± 20*	2.6
SIN-1 (30 nM)	3	232 ± 31	74 ± 4**	3.1
Cilostamide (20 nM)	3	251 ± 59	68 ± 6*	3.7
Ro 20-1724 (10 μM)	3	193 ± 45	101 ± 25**	1.9

Table 4.2. Effects of low concentrations of nitrovasodilators and of inhibitors of cAMP phosphodiesterase on the ability of isoproterenol to inhibit the contraction of rat aortic rings by 100 nM phenylephrine

Isoproterenol was added without or with each of the listed compounds 30 s before phenylephrine and the contraction of the aortic rings was measured after a further 2 min. In each experiment, IC_{50} values for isoproterenol in the absence and presence of one of the compounds listed were calculated from concentration-response curves obtained from the same aortic rings, as described under Section 2.2.18. Results are means ± S.E.M. from the numbers of experiments shown; the significance of changes in IC_{50} values for isoproterenol were evaluated by two-sided paired _t_ tests after logarithmic transformation of the results; $^{*}p < 0.02$, $^{**}p < 0.001$. The ratio of IC_{50} values for isoproterenol in the absence or presence of other drugs indicates the extent of synergism.

Compounds studied	No. of expts.	IC_{50} of isoproterenol (nM)		Ratio of IC_{50} values
		Without compound	With compound	
SNP (5 nM)	5	334 ± 49	37 ± 5**	9.0
SIN-1 (1 μM)	3	398 ± 55	42 ± 10*	9.5
Cilostamide (1 μM)	3	318 ± 57	34 ± 4*	9.4
Ro 20-1724 (100 μM)	4	356 ± 57	44 ± 3**	8.1

Table 4.3. Effects of 50 nM SNP and 500 nM isoproterenol on cyclic nucleotide levels in rat aortic smooth muscle

Rat aortic rings were incubated for 2.5 min with SNP and isoproterenol, singly or in combination, before determination of tissue cAMP and cGMP. Values are means ± S.E.M. from 6-8 separate incubations, each containing 2 aortic rings. Significant increases are shown; $^{*}p < 0.02$; $^{**}p < 0.001$.

Additions	cAMP (pmol/mg of protein)	cGMP (pmol/mg of protein)
None	0.58 ± 0.04	0.17 ± 0.05
SNP (50 nM)	1.01 ± 0.14*	0.80 ± 0.10**
isoproterenol (500 nM)	0.98 ± 0.13*	0.19 ± 0.06
SNP (50 nM) + isoproterenol (500 nM)	3.01 ± 0.40**	0.87 ± 0.13**

Chapter 5: *Agonist-induced changes in cyclic nucleotides in cultured VSMC*

5.1. *Introduction*

Cultured VSMC have been used to study many different questions relating to VSM structure, growth, physiology, electrophysiology, pharmacology and gentics (Lieberman *et al.*, 1987). Compared to pieces of isolated vascular tissue, cultured VSMC offer a variety of advantages. Cultured VSMC can contain only one cell type and the medium in which the cells are grown is easily changed. Also, the diffusional barriers which exist in intact vascular tissue are removed when cells are grown in culture. Changes in many different pharmacological and biochemical parameters have been studied in cultured VSMC and cultured cells from other types of muscle (Lieberman *et al.*, 1987).

Changes in the cyclic nucleotide levels of isolated pieces of VSM can be measured by radioimmunoassays (Harper and Brooker, 1975). However, these assays are time consuming and expensive. In addition, substantial variability arises because of the difficulties involved in generating comparable tissue samples. In principle, the effects of drugs on the levels of cyclic nucleotides in smooth muscle can be studied much more easily using cultured VSMC, grown from extracts of the tissue of interest. Changes in the levels of cyclic nucleotides have been measured in cultured VSMC cells using radioimmunoassays (Sands *et*

al., 1976; Nabika, *et al.*, 1985; Nakamura *et al.*, 1988; Resnik *et al.*, 1989; Hamet *et al.*, 1989). However, because the levels of both cyclic nucleotides in unstimulated VSMC are very low, large numbers of cells were needed to obtain reliable estimates using this method.

5.1.1. *Objectives*

One of the important objectives of this part of my thesis was to develop a prelabelling method that would allow for the simultaneous measurements of cAMP and cGMP in cultured VSMC. This method was necessary in order to assess whether a cultured VSMC system could be used to study interactions between nitrovasodilators and activators of adenylyl cyclase, similar those described in platelets (Chapter 3) and in rat aortic rings (Chapter 4).

5.2. *Results*

5.2.1. *Measurement of cyclic nucleotides in cultured VSMC*

In preliminary experiments, aimed at developing a prelabelling assay for the measurement of cyclic nucleotides in cultured VSMC, difficulties arose because of divergent responses obtained with cultures derived from different rat aortae. In these early experiments, five distinct cultures, all derived from different WKY-rat aortae, were

labelled with [^{3}H]adenine and the changes in [^{3}H]cAMP levels in response to different agents were measured. These cells were referred to as W1, W2, W3, W4 and W5, respectively, based on a nomenclature system established by Dr. R.M.K.W. Lee. Experiments with these five cell lines indicated that one of them (W1) responded differently to activators of adenylyl cyclase and was thus pharmacologically distinct (Table 5.1). In these experiments, isoproterenol increased the level of [^{3}H]cAMP only in the W1 cell line, whereas adenosine had no effect on the [^{3}H]cAMP in W1 cells but increased [^{3}H]cAMP of the other four cell lines (Table 5.1). Consistent with these results, Drs. Lee and Kwan have found that W1 cells have a much larger number of β-receptors than any of the other WKY cultured cell lines (personal communication). These results may reflect variability in the β-receptor density of aortae taken from different WKY-rats. Such variability is possible, given the genetic heterogeneity of WKY rats (Kurtz *et al.*, 1989). Although changes in receptor expression during culture may also have contributed to these results, another explanation is also possible. Thus, each of the WKY cultured cell lines (W1-W5) was established in parallel with an SHR VSMC culture. It is therefore theoretically possible that in the case of the W1 cells, the WKY and SHR cultures were "mixed-up" at an early stage. Consistent with this is the fact that with the exception of the W1 cells, all the SHR cell lines derived from these paired experiments had higher β-receptor densities than their respective WKY paired culture (Kwan and Lee, 1990). In these early experiments, VSMC were only labelled with [^{3}H]adenine, and changes in [^{3}H]cGMP in response to

nitrovasodilators were not measured. Since both isoproterenol and adenosine are known to cause relaxation of VSMC, a series of experiments were undertaken using cells in which incubation with isoproterenol (W1), or adenosine (W2), caused significant increases in [^{3}H]cAMP (see Table 5.5). However, in these latter experiments, the VSMC were labelled with [^{3}H]hypoxanthine, which permitted the labelling of both the ATP and GTP pools of these cell lines (Section 5.2.2.). The general characteristics of these two cell lines are summarized in Table 5.2.

5.2.2. *Labelling of cultured VSMC by [^{3}H]hypoxanthine*

Preliminary experiments showed that it was not possible to label the cultured VSMC using the methods that had been established for washed rabbit or rat platelets. Although the uptake of [^{3}H]adenine by cultured VSMC was sufficient to permit determination of changes in [^{3}H]cAMP (see Table 5.1), these cells did not incorporate sufficient [^{3}H]guanosine to allow changes in [^{3}H]cGMP to be measured. Thus, at a labelling concentration of 2 μM the uptake of [^{3}H]guanosine by the cultured VSMC was less than 5% after 24 h. [^{3}H]guanosine was used because [^{3}H]guanine was no longer commercially available at the time of these experiments. Therefore, a different prelabelling method was established to permit the simultaneous measurement of changes in [^{3}H]cAMP and [^{3}H]cGMP, without use of either [^{3}H]guanine or [^{3}H]guanosine.

In many cells, including smooth muscle, hypoxanthine can be

converted to inosine monophosphate (IMP) by hypoxanthine-guanine phosphoribosyl transferase (Fig. 5.1). Since IMP is a common metabolic precursor for the synthesis of both AMP and GMP, incorporation of sufficient amounts of [^{3}H]hypoxanthine by the cultured VSMC should allow both [^{3}H]ATP and [^{3}H]GTP to be formed. Both W1 and W2 cultured VSMC incorporated [^{3}H]hypoxanthine in a time dependent manner (Fig. 5.2). Thus, the uptake of [^{3}H]hypoxanthine by W1 cultured VSMC amounted to 54 $\pm$ 7% after 16h (mean $\pm$ S.E.M. from 7 experiments). In these cells, 32 $\pm$ 4% (mean $\pm$ S.E.M.) of the [^{3}H]hypoxanthine taken up was incorporated into [^{3}H]ATP as determined by two-dimensional t.l.c. (Section 2.2.11.). Thus, after a 16 h incubation with 10 μCi of [^{3}H]hypoxanthine, approximately 4 x 10^6 d.p.m. of [^{3}H]ATP had accumulated in each sample (well) of cultured cells, an amount equivalent to 4 x 10^7 d.p.m./mg of protein. In these cells, a much lower percentage of the [^{3}H]hypoxanthine was incorporated into the GTP pool. Thus, 3 $\pm$ 1% (mean $\pm$ S.E.M. from 7 experiments) of the [^{3}H]hypoxanthine taken up by these cells was converted to [^{3}H]GTP. On average, the incorporation of label into the GTP pool resulted in the accumulation of 3 x 10^5 d.p.m. of [^{3}H]GTP per well of cells. This amount of [^{3}H]GTP was just sufficient to allow an estimation of the basal [^{3}H]cGMP present in unstimulated cells and made it possible to determine accurately the amounts of [^{3}H]cGMP formed in response to compounds that activated guanylyl cyclase (Table 5.4.). Since both ITP and XTP are metabolic intermediates in the biosynthesis of ATP and GTP from hypoxanthine (Fig. 5.1), the incorporation of [^{3}H]hypoxanthine

into each of these compounds was also monitored. This value was << 1% (mean from 2 experiments). The amount of [^{3}H]hypoxanthine incorporated into the acid precipitate, which presumably contained most of the DNA and RNA, accounted for 13% of the [^{3}H]hypoxanthine taken up by the cultured cells (mean from 2 experiments).

The amount of [^{3}H]hypoxanthine taken up by W2 cells (39 ± 5%, in 16-18 h, mean ± S.E.M. from 6 experiments) was not significantly different from that incorporated by W1 cells. The percent [^{3}H]ATP and [^{3}H]GTP formed from [^{3}H]hypoxanthine in the W2 cultured VSMC were also similar to those formed in W1 cells. Thus, when W2 cells were incubated with [^{3}H]hypoxanthine for 16-18 h, 34 ± 7% and 2 ± 0% (means ± S.E.M. from 6 experiments) of the radioactivity accumulated in the [^{3}H]ATP and [^{3}H]GTP pools, respectively.

5.2.3. *Radioisotopic purity of cyclic nucleotides isolated from prelabelled cultured VSMC*

The purity of the cyclic [^{3}H]nucleotides isolated by the dual column procedure used to process samples from these types of experiments (Section 2.2.8) was assessed by a further purification by t.l.c. (Section 2.2.9.). Since the cyclic [^{14}C]nucleotides added to these samples was pure, any reduction in the ratio of ^{3}H/^{14}C must reflect ^{3}H-labelled compounds that did not co-chromatograph with the cyclic [^{14}C]nucleotide. Results of such an experiment, using samples derived

from W1 cells, are shown (Table 5.3.). The results indicated that the [^{3}H]cGMP isolated from unstimulated cells by the two-column method was approximately 75% pure, whereas no significant impurities were detected in the [^{3}H]cGMP isolated from cells stimulated with 10 μM SNP. Similar results were obtained when the purity of [^{3}H]cGMP isolated from rabbit platelets was assessed (see Table 3.1). The radioisotopic purity of [^{3}H]cAMP isolated by the dual-column method from both stimulated and unstimulated cells was > 90 % (Table 5.3.).

5.2.4. *Validation of the prelabelling assay using radioimmunoassays*

A direct comparison of results obtained from parallel prelabelling assay and radioimmunoassays was used to validate the prelabelling assay in W1 cells. Under all experimental conditions, results obtained with both methods gave virtually identical results (Table 5.4.). Isoproterenol (10 μM) increased the level of cAMP by 140-fold in 1 min, as measured by the prelabelling assay, whereas the increase measured by radioimmunoassay was 119-fold; these values were not significantly different. Similarly, the increases in cGMP in cultured VSMC caused by SNP (10 μM) were 352-fold and 349-fold, as determined by prelabelling assays and radioimmunoassays, respectively. Thus, results obtained by these two methods were not different. Also, the results indicate that overnight incubation of VSMC with [^{3}H]hypoxanthine causes homogeneous labelling of both the adenine and the guanine nucleotide pools, since no attempt was made to isolate

metabolically active pools of [^{3}H]ATP and [^{3}H]GTP from the prelabelled cells for the determination of the specific activities of the cyclic nucleotide precursors.

In marked contrast to results obtained in platelets and rat aortic rings, incubation of the cultured VSMC with SNP in this experiment did not increase the level of [^{3}H]cAMP above its basal value, whether cAMP was measured by the prelabelling or radioimmunoassay (Table 5.4.). In fact, further studies showed that in 8 prelabelling experiments with W1 cells, SNP (10 μM) had no significant effect on the level of [^{3}H]cAMP (Table 5.5.).

5.2.5. *Effects of isoproterenol on cyclic nucleotide levels in cultured VSMC (W1)*

Isoproterenol caused concentration-dependent increases in the levels of [^{3}H]cAMP in W1 cultured VSMC (Fig. 5.3). Basal [^{3}H]cAMP in these cells was 0.08 ± 0.01% of [^{3}H]ATP (mean ± S.E.M. from 8 experiments). This value was substantially higher than that observed in prelabelled rabbit platelets, but similar to that recorded for prelabelled rat platelets (see Chapter 3). Relative to the basal value, the maximum increase in [^{3}H]cAMP caused by isoproterenol after 1 min was, on average, equivalent to a 40-fold increase in this cyclic nucleotide. However, the increases in [^{3}H]cAMP in different experiments were quite variable, ranging from 25- to 90-fold. The

concentration of isoproterenol found to cause half-maximal stimulation of [^{3}H]cAMP formation was 102 ± 29 nM (mean ± S.E.M. from 3 experiments).

Analysis of the time course of the increases in [^{3}H]cAMP caused by isoproterenol indicated that the maximum increase occurred after about 1 min (Fig. 5.4.). The [^{3}H]cAMP level then decreased slowly but was still substantially higher than basal values after 3 min. Isoproterenol did not affect either the basal or the SNP-induced increases in [^{3}H]cGMP (Table 5.5.).

5.2.6. *Effects of SNP on cyclic nucleotide levels in cultured VSMC (W1)*

The basal level of [^{3}H]cGMP in these cells was equivalent to 0.07 ± 0.01% of the [^{3}H]GTP (mean ± S.E.M. from 8 experiments). This value for [^{3}H]cGMP is approximately 6-fold higher than that obtained in prelabelled rabbit or rat platelets. Determination of mass amounts of cGMP in these cells also gave a value 6-fold higher in the cultured VSMC than in the platelets. The nitrovasodilator, SNP, caused concentration-dependent increases in [^{3}H]cGMP (Fig. 5.5.). These SNP-induced increases in [^{3}H]cGMP did not appear to reach a maximum even with a concentration as high as 100 μM. Incubation of these cells with 10 μM SNP for 1 min caused, on average, a 70-fold increase in the level of [^{3}H]cGMP (mean value from 8 experiments). However, as with the isoproterenol-induced increases in [^{3}H]cAMP in

these cells, the increases in $[^3H]$cGMP caused by SNP in different experiments were quite variable and ranged from 25- to 180-fold of the unstimulated value.

Analysis of the time course of the increases in $[^3H]$cGMP in VSMC caused by SNP showed that this cyclic nucleotide continued to increase for at least 3 min, the longest period studied (Fig. 5.6). These results contrast with those obtained in platelets, in which the SNP-induced increases in $[^3H]$cGMP peaked within 1 min and then returned towards basal values. Also, SNP did not affect the basal level of $[^3H]$cAMP, or affect the increases in $[^3H]$cAMP caused by isoproterenol in these cells (Table 5.5).

5.2.7. *Effects of SNP, cilostamide and Ro 20-1724 on isoproterenol-induced increases in $[^3H]$cAMP in VSMC (W1)*

In marked contrast to the results obtained in either rabbit or rat platelets (Chapter 3), and in intact aortic smooth muscle (Chapter 4), SNP did not increase basal $[^3H]$cAMP values or potentiate the increases in $[^3H]$cAMP caused by isoproterenol in W1 cells (Tables 5.4, 5.5 and 5.6). Consistent with the implications of these findings, cilostamide, a selective inhibitor of the cGI-PDE, also had no effect on either basal or isoproterenol-stimulated $[^3H]$cAMP levels in these cells (Table 5.6). The concentration of cilostamide used in these experiments (10 μM) was sufficient to increase basal $[^3H]$cAMP and

potentiate markedly the increases in this cyclic nucleotide caused by activation of adenylyl cyclase in platelets (Chapter 3). In contrast, 300 μM Ro 20-1724, a compound known to inhibit another PDE (RoI-PDE), increased basal [^{3}H]cAMP by 43 ± 7% (mean value ± S.E.M. from 4 experiments) in W1 cultured VSMC and doubled the increase in [^{3}H]cAMP caused by 100 μM isoproterenol (mean from 2 experiments). The effects of Ro 20-1724 and isoproterenol were supra-additive ($p < 0.005$). These data strongly imply that cGI-PDE is absent from these cultured VSMC, whereas the RoI-PDE is present. Inhibition of the latter PDE by Ro 20-1724 presumably potentiates the effects of isoproterenol.

5.2.8. *Effects of adenosine on the cyclic nucleotide levels cultured VSMC (W2)*

Although isoproterenol did not affect the [^{3}H]cAMP levels in W2 cells (Table 5.1), adenosine caused concentration-dependent increases in this cyclic nucleotide (Fig. 5.7.). The basal level of [^{3}H]cAMP in these cells was 0.08 ± 0.02% of the [^{3}H]ATP (mean ± S.E.M. from 3 experiments). The maximum increase in [^{3}H]cAMP was obtained with 100 μM adenosine, and in some experiments reached levels 4-fold higher than the basal values after 1 min (Fig. 5.7.; Table 5.5.). Adenosine had no effect on [^{3}H]cGMP in these cells (Table 5.5).

The time course of the adenosine-induced increase in [^{3}H]cAMP is shown in Fig. 5.8. Thus, adenosine (100 μM) caused a maximum increases in [^{3}H]cAMP by 0.5 min. In the continued presence of 100 μM adenosine, the [^{3}H]cAMP remained at this elevated level for at least 3 min, the longest time period investigated (Fig. 5.8.).

5.2.9. *Effects of SNP on cyclic nucleotide levels in cultured VSMC (W2)*

The amount of [^{3}H]GTP formed from [^{3}H]hypoxanthine in the W2 cultured VSMC line was similar to that obtained in the W1 cell line. However, the basal level of [^{3}H]cGMP in these cells (0.03 $\pm$ 0.01%, mean $\pm$ S.E.M. from 3 experiments) was substantially lower in these cells than that measured in the W1 VSMC cultures. Also, concentrations of SNP as high as 100 μM had no effect on the level of [^{3}H]cGMP in the W2 cultured cells (Table 5.5). Whether the inability of SNP to bring about an increase in [^{3}H]cGMP in these cells (W2) is a result of the absence of soluble guanylyl cyclase activity or due to other factors is still unclear.

5.2.10. *Effects of SNP, cilostamide and Ro 20-1724 on adenosine-induced increases in [^{3}H]cAMP in VSMC (W2)*

Neither SNP nor cilostamide caused significant increases in [^{3}H]cAMP in W2 cells in the presence or absence of adenosine (Table

5.7). Since the mechanism by which SNP increases platelet and smooth muscle cAMP involves inhibition of cAMP breakdown by cGMP (Chapters 3 and 4), the failure of SNP to increase [^{3}H]cAMP could be accounted for by the lack of a cGMP response to SNP in these cells. However, the lack of a cilostamide-induced increase in the [^{3}H]cAMP in these cells implies that cGI-PDE is also absent. As with W1 cultured VSMC, Ro 20-1724 (300 μM) caused a significant increase [^{3}H]cAMP in W2 cells when incubated alone with the cells and also potentiated the agonist (in this case adenosine)-induced increases in [^{3}H]cAMP in these cells (Table 5.7). The effects of Ro 20-1724 and adenosine were supra-additive ($p < 0.005$).

5.3. *Discussion*

Although changes in the levels of cAMP and cGMP, resulting from the actions of drugs or hormones, can be measured by radioimmunoassays, these assays are time-consuming and expensive. An alternative method involves pre-incubation of cells with labelled adenine and guanine or guanosine and, following incorporation of radioactivity into cellular ATP and GTP, measurement of changes in labelled cAMP and cGMP. Changes in [^{3}H]cAMP in platelets following pre-incubation with ^{3}H-labelled adenine have been widely studied (e.g. Haslam and McClenaghan, 1981). Similarly, changes in platelet [^{3}H]cGMP have also been determined following incubation of platelets with [^{3}H]guanine (Davies *et al.*, 1976). Moreover, these prelabelling assays have been successfully

combined, to permit simultaneous determination of cyclic nucleotides in prelabelled platelets (see Chapter 2). In this chapter, I described a prelabelling method using [^{3}H]hypoxanthine that permits the rapid and reliable measurement of changes in both cAMP and cGMP in small numbers of cultured VSMC. It was necessary to use [^{3}H]hypoxanthine to label the VSMC, instead of [^{3}H]adenine and [^{3}H]guanine, for two reasons. First, the [^{3}H]guanine that had been successfully used to label the rabbit platelet GTP pool, was no longer commercially available and second, [^{3}H]guanosine, though satisfactory for labelled rat platelets (Chapter 3), was not incorporated into VSMC in sufficient amounts. The prelabelling assays using [^{3}H]hypoxanthine were validated using commercially available radioimmunoassays for the two cyclic nucleotides. Virtually identical results were obtained. However, estimation of changes in either cyclic nucleotide by radioimmunoassay, required 4-fold more cells than were required using the prelabelling assay.

The effects of SNP, isoproterenol and adenosine on cGMP and cAMP in cultured VSMC were studied using the prelabelling assay. As discussed previously, it is generally accepted that the effects of nitrovasodilators, on VSMC are mediated by the increases in cGMP that result from the activation of soluble guanylyl cyclase (Murad, 1986; Lincoln, 1990). Similarly, the effects of isoproterenol and adenosine are thought to be mediated by increases in cAMP (Hardman, 1984; Murray, 1990). The results obtained in the present study confirm earlier

reports of the effects of these compounds on the cyclic nucleotide levels of VSMC (reviewed in Murray, 1990, and Lincoln, 1990). Thus, in one cell line (W1), isoproterenol increased cAMP in a time and concentration dependent manner. The effects of this β-agonist on the cAMP content of these cells, as determined by the prelabelling assay, were very similar to those obtained by Nabika *et al.* (1985) in cultured WKY- and SHR-rat aortic VSMC by radioimmunoassay. In the earlier report, the basal value of cAMP was found to be 18 $\pm$ 2 pmol/mg of protein, slightly higher than that of 10 $\pm$ 1 pmol/mg of protein found in the present study. In addition, the increases in cAMP caused by isoproterenol in both studies were very similar. The basal value of cGMP was also measured by Nabika *et al.* (1985) and found to be 2 $\pm$ 1 pmol/mg of protein, somewhat less than the value of 6 $\pm$ 1 pmol/mg of protein obtained with W1 cells.

In another VSMC line, shown to be unresponsive to isoproterenol (W2), adenosine caused concentration-dependent increases in cAMP. This is consistent with the hypothesis that adenosine causes VSM relaxation via the activation of smooth muscle adenylyl cyclase (Kukovetz *et al.*, 1978). Adenosine may mediate this effect via interaction with A_2-adenosine receptors, which have previously been demonstrated on cultured VSMC from rats (Anand-Srivastava *et al.*, 1982). Recent reports of an endothelial component to the action of adenosine in causing vascular relaxation (Moritoki *et al.*, 1990; Headrick and Berne, 1990) may help to explain some earlier work that casts doubt on

the importance of cAMP in the relaxation caused by this compound (Herlihy *et al.*, 1976; Verhaeghe, 1977). Thus, the presence of a healthy endothelial cell layer, and its ability to release EDRF, must be considered in relation to the effects of adenosine on vascular tone. Moreover, since the effects of adenosine on cAMP in cultured VSMC (this Chapter) were relatively small and difficult to detect in intact aorta (Moritoki *et al.*, 1990), endothelial release of EDRF may be a very important component of the physiological relaxation of VSM by adenosine (see Chapter 4).

One unexpected result was that concentrations of SNP as high as 100 μM had no effect on the basal cGMP levels of the adenosine-responsive VSMC. Although other explanations are possible, the most likely reason is that this cultured VSMC line (W2) may have lost the ability to express soluble guanylyl cyclase during culture. The loss of selected proteins (receptors and enzymes) during cell culture is a well-established phenomenon, and has previously been documented for the cGMP-PK in rat aortic cultured VSMC (Lincoln *et al.*, 1990). However, loss of guanylyl cyclase activity is clearly not an inevitable consequence of growing these cells in culture, since SNP did stimulate cGMP formation in W1 cells. Whether these cells express the particulate form of guanylyl cyclase has not yet been studied. It may be of interest that BALB/c3T3 fibroblast also lack soluble guanylyl cyclase (Nesbitt *et al.*, 1976).

In the present experiments, special attention was given to the changes in cAMP that occurred when SNP was used in combination with either isoproterenol or adenosine. In platelets, co-incubation with nitrovasodilators and activators of adenylyl cyclase was shown to cause markedly larger increases in cAMP than were obtained with either agent alone (Chapter 3). Moreover, evidence was obtained that the potentiation of the effects of the activators of adenylyl cyclase by the nitrovasodilators was attributable to inhibition of cGI-PDE by cGMP. However, in experiments with W1 cultured VSMC, no synergistic increase in the level of cAMP was noted when these cells were incubated simultaneously with both SNP and isoproterenol, despite the fact that SNP caused marked increases in the level of cGMP in these cells. Similar results were obtained with cilostamide, a compound known to inhibit the cGI-PDE. Cilostamide, at concentrations as high as 10 μM, did not increase basal cAMP, or potentiate the increases in cAMP caused by isoproterenol in W1 cells or by adenosine in W2 cells. The effects of both SNP and cilostamide in these cells are in marked contrast to the effects of these compounds on intact rat aorta (Chapter 4). Thus, these results are consistent with the suggestion that the cGI-PDE is absent, or inactive, in both of these cell lines.

Ro 20-1724, an inhibitor of a low K_m cAMP-PDE (RoI-PDE), increased basal cAMP and potentiated the increases in cAMP caused by isoproterenol and adenosine in W1 and W2 cells, respectively. These results are consistent with the effects of this compound on rat aortic

smooth muscle reported by Schoeffter *et al.* (1987) and in this thesis (Chapter 4; Maurice *et al.*, 1990). Also, these results show that the PDE inhibited by Ro 20-1724 was not lost during culture of these VSMC. The selective loss of one PDE activity over the other in both cell lines studied may imply a differential transcriptional regulation of these two enzymes.

Although isoproterenol and SNP increased cAMP and cGMP, respectively, in W1 cells, and adenosine increased the level of cAMP in W2 cells, the significance of each of these effects, and their relevance to signal transduction mechanisms in VSMC, cannot be assessed until the reason(s) for the differences between the two cell lines (W1 and W2), have been elucidated. One possible explanation, as discussed previously (Section 5.2.1), is that W1 cells are not derived from WKY VSMC but from SHR VSMC, as a result of a "mix-up" or contamination. This suggestion is based on comparison of other pairs of cultured WKY and SHR cells lines established in Dr. Lee's laboratory. Thus, radioligand binding experiments (Kwan and Lee, 1990) have indicated that cultured WKY-rat aortic VSMC have fewer β-receptors than paired SHR-rat aortic VSMC. However, Nabika *et al.* (1985) have reported that isoproterenol can cause substantial increases in the level of cAMP in cultured VSMC from both WKY and SHR rat aortae. In addition, these authors found no differences in the concentration-response curves for isoproterenol in the WKY and SHR cultured VSMC. Experiments with SHR cells, similar to those described in this Chapter, should help in the interpretation of

the discrepant results obtained with the W1 and W2 cell lines. Moreover, studies with very early passages of W1 and W2 cells should indicate the stage at which guanylyl cyclase is lost in W2 cells and cGI-PDE is lost in both cell lines. However, since DNA fingerprinting studies have shown that WKY rats are genetically heterogeneous, the results obtained in this study could have resulted from variability in the genotype of the animals obtained from the supplier. DNA fingerprinting studies with the cells used in this study could answer this question. My results cast doubt on the value of cultured aortic VSMC as a model for the analysis of signal transduction mechanisms in the VSM from which they are derived.

5.4 *Summary*

5.4.1. A fast, reliable and accurate prelabelling assay using $[^{3}H]$hypoxanthine was developed to permit the simultaneous measurement of changes both cGMP and cAMP in cultured VSMC.

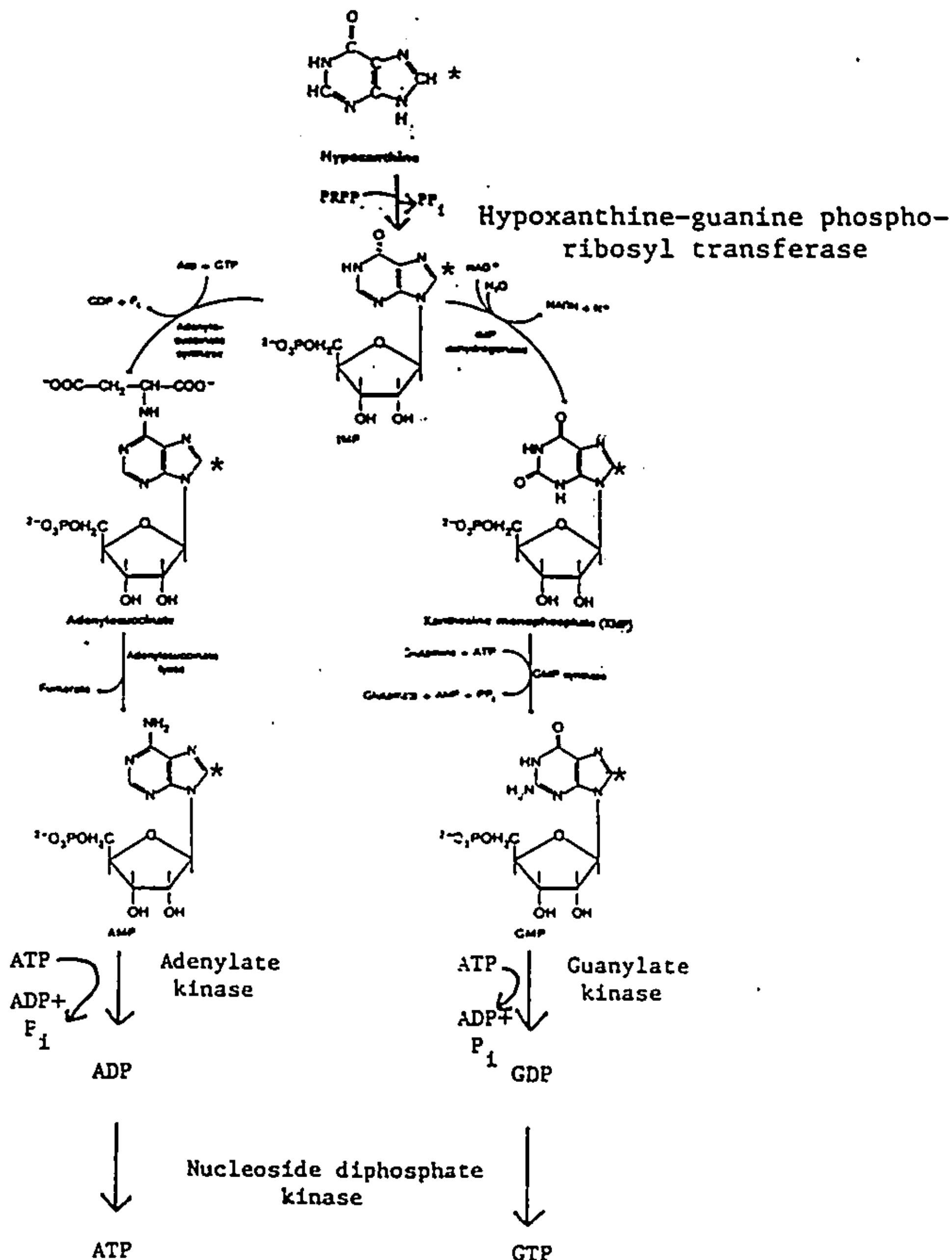
5.4.2. Using this assay, I investigated the changes in both cGMP and cAMP in two cultured VSMC lines, caused by their incubation with isoproterenol, adenosine or SNP. In one line (W1 cells), time and concentration-dependent increases in cAMP were observed upon incubation of the cells with isoproterenol. In the second line (W2 cells), which was unresponsive to isoproterenol, adenosine caused concentration-dependent increases in cAMP. In the isoproterenol-responsive cells, but not in the adenosine responsive cells, SNP caused concentration-dependent increases in cGMP.

5.4.3. In neither cell line did SNP cause an increase in cAMP or enhance the increases in cAMP caused by isoproterenol or adenosine. The results suggest that cGI-PDE activity was absent from the isoproterenol-responsive cells and that both soluble guanylyl cyclase activity and cGI-PDE were absent from the adenosine-responsive cells.

5.4.4. Ro 20-1724, an inhibitor of a low K_m cAMP PDE that is not inhibited by cGMP, increased cAMP in both cultured VSMC lines and potentiated the increases caused by isoproterenol or adenosine.

5.4.5. I conclude that prelabelling with [^{3}H]hypoxanthine permits rapid analysis of the effects of compounds that increase cyclic nucleotide levels in cultured VSMC. However, it was apparent that cultured VSMC differ significantly in their responses from the VSM from which they are derived, as a result of the selective loss of receptors and enzymes that participate in signal transduction.

Fig. 5.1. Metabolic pathway of ATP and GTP formation from $[^3H]$ hypoxanthine

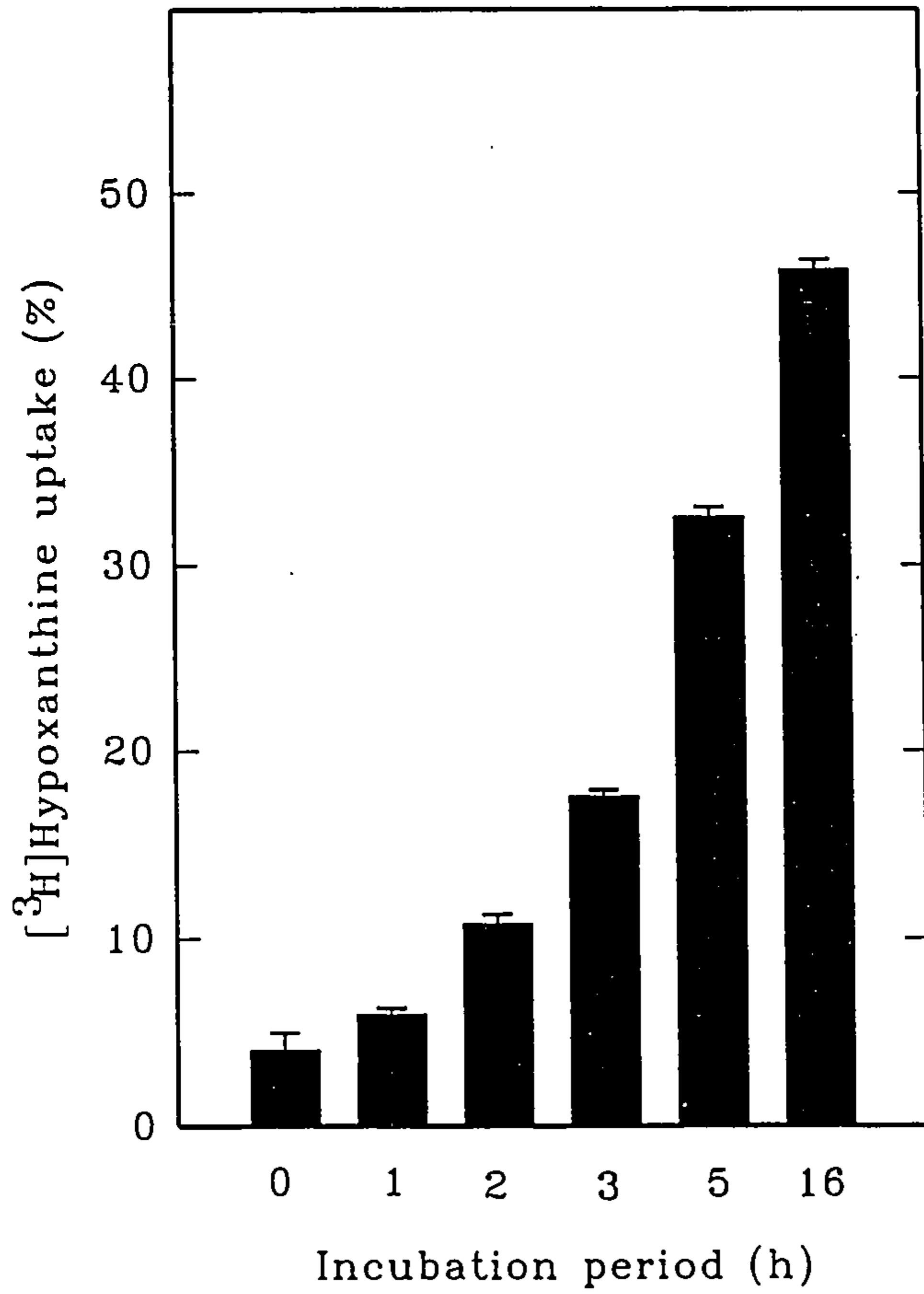


PRPP: Phosphoribosylpyrophosphate
PP_i : Pyrophosphate
NAD^+: Nicotinamide adenine dinucleotide
* : Label (^3H)

Modified from: Biochemistry 2nd Edition, G. Zubay
Collier Macmillan Canada, Inc.

Fig. 5.2. Time course of [^{3}H]hypoxanthine uptake by cultured rat aortic VSMC

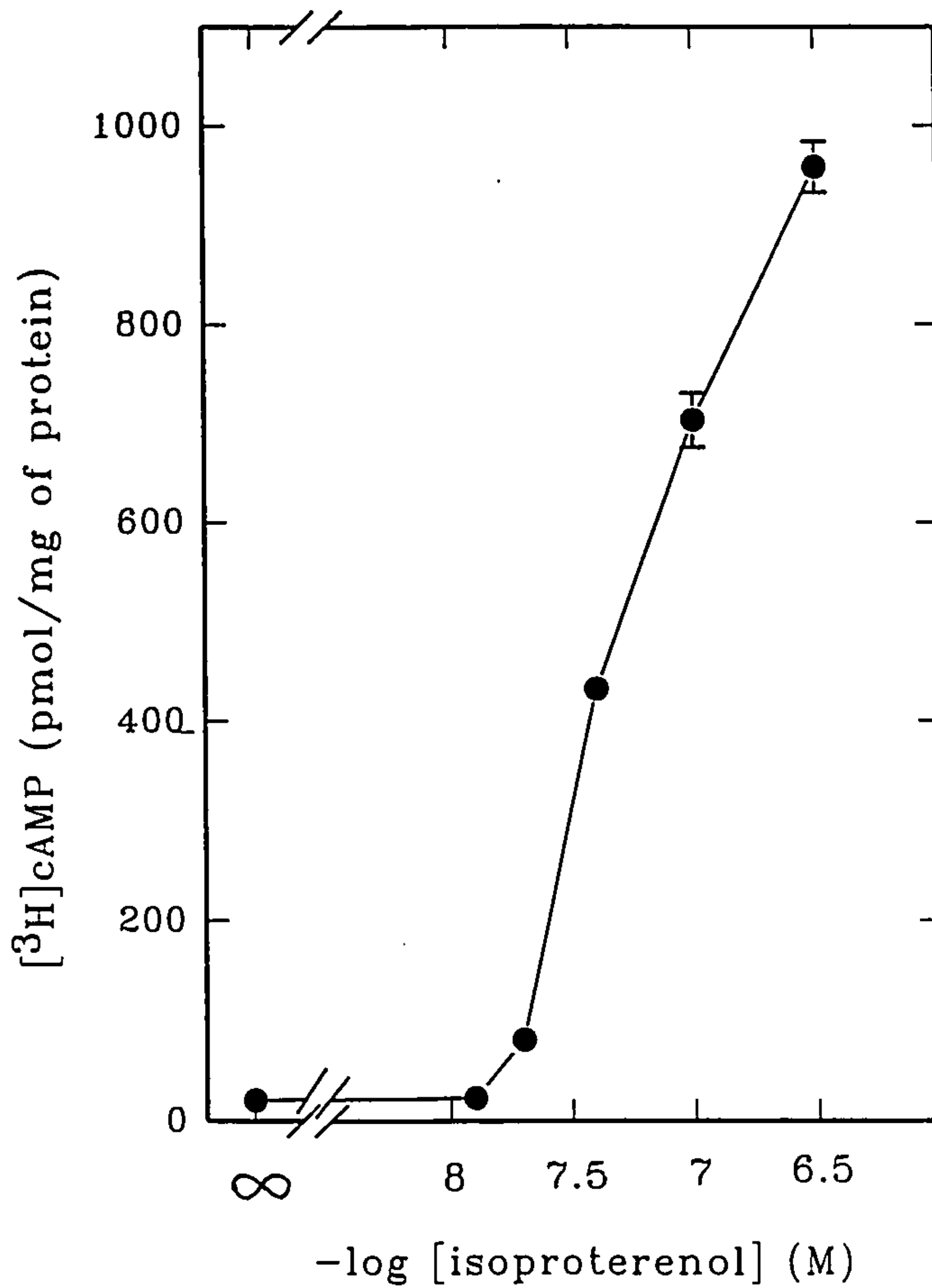
Cultured rat aortic VSMC were grown in 24 well culture dishes until confluent. The cells were then covered with fresh DMEM containing [^{3}H]hypoxanthine (10 Ci/mmol) at a final concentration of 2 μM. The cells were incubated at 37°C in a controlled atmosphere of 95% air/5% CO_2 for the indicated times. Following removal of the labelling medium, the cells were washed twice with fresh DMEM (without [^{3}H]hypoxanthine) and then extracted with 0.5 ml of trichloroacetic acid (5% w/v final concentration). The precipitated protein and acidified extract were transferred to polypropylene tubes and the protein separated by centrifugation for 2 min at 12,000 x g. The percent incorporation was calculated by counting a fixed volume of the acidified extract. Values are means ± S.E.M. from 3 individual determinations in the same experiment.



[3H]Hypoxanthine uptake (%)
0
10
20
30
40
50
0
1
2
3
5
16
Incubation period (h)

Fig. 5.3. Effects of different concentrations of isoproterenol on the [^{3}H]cAMP level of cultured VSMC

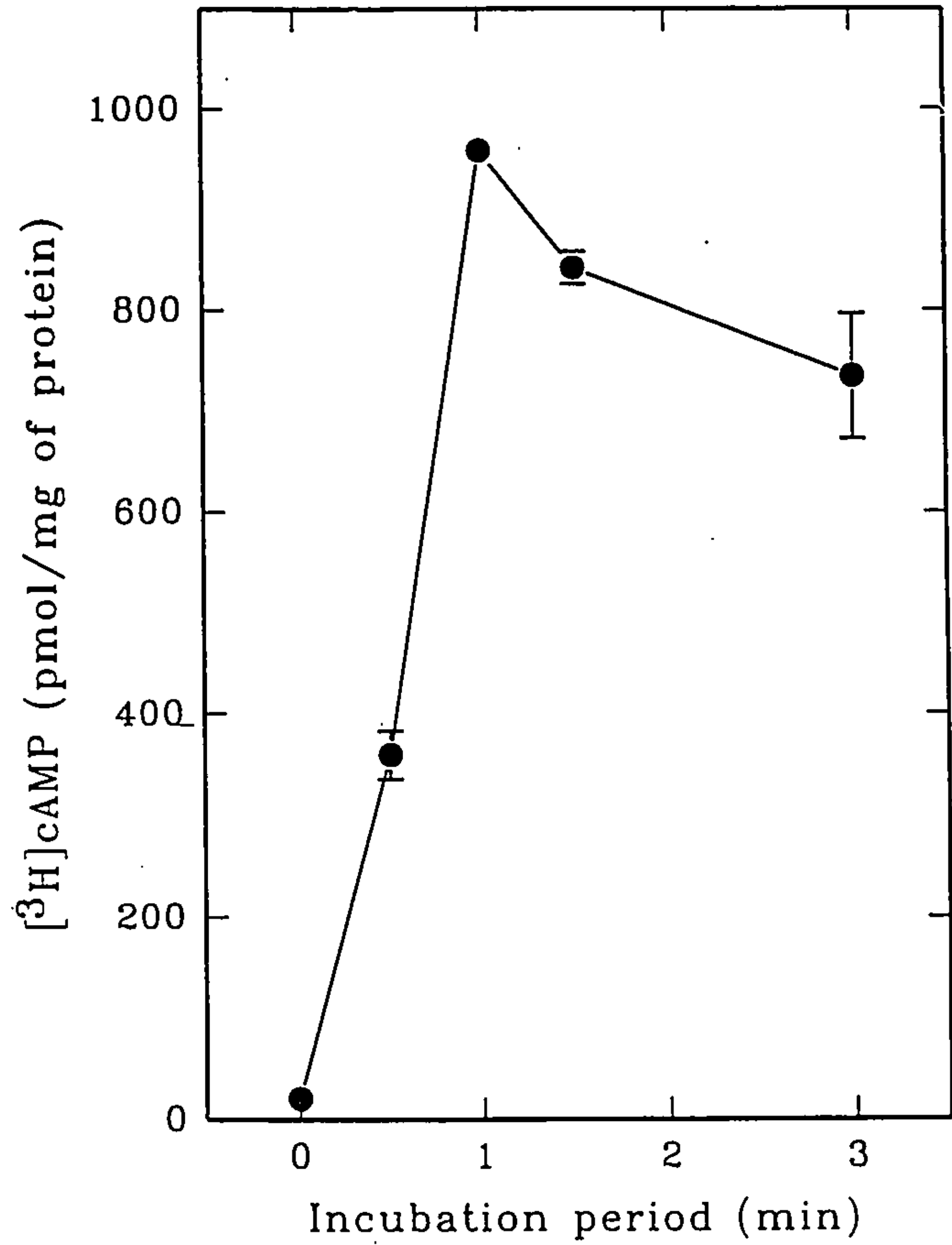
Cultured VSMC were grown in 24 well dishes until confluent and then labelled overnight with [^{3}H]hypoxanthine (10 Ci/mmol; 2 μM). Following removal of the labelling medium, the cells were washed three times with HBSS (37°) and then covered with 0.45 ml of fresh HBSS at 37 ^{o}C. Cells were incubated at 37° for 1 min with saline (C) or isoproterenol. Incubations were terminated by addition of ice-cold trichloroacetic acid (final concentration 5% (w/v)). The precipitated protein and the acidified extract were transferred to polypropylene tubes and centrifuged at 12,000 x g for 2 min. cAMP was expressed as pmol/mg of protein using a value for the specific activity for [^{3}H]ATP determined as in Section 2.2.11. Values are means $\pm$ S.E.M. from 3 determinations.



1000
800
600
400
200
0
[3H]cAMP (pmol/mg of protein)
∞
8
7.5
7
6.5
−log [isoproterenol] (M)

Fig. 5.4. Time course of isoproterenol-induced increases in [^{3}H]cAMP in cultured VSMC

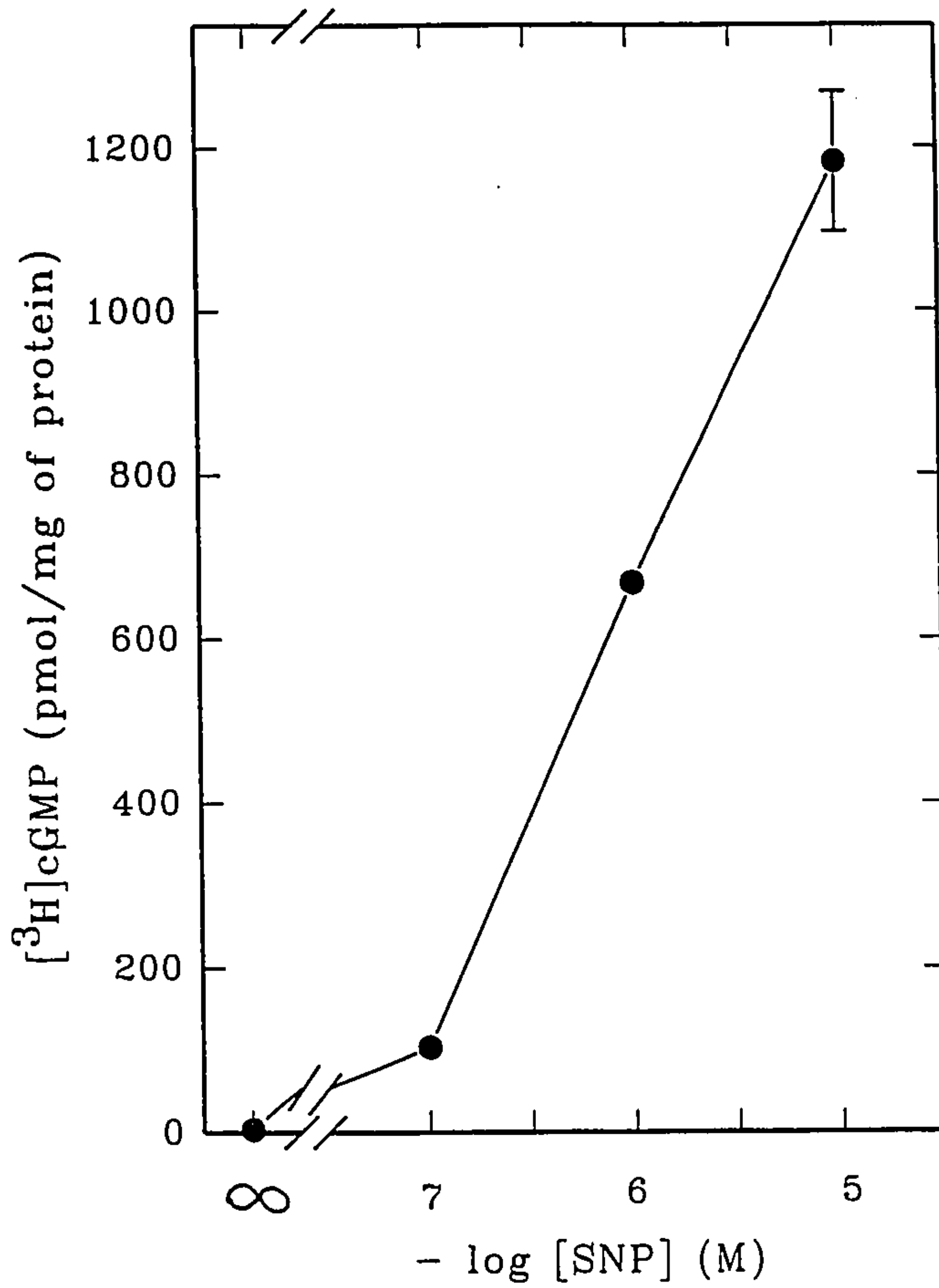
Cultured VSMC were labelled with [^{3}H]hypoxanthine (Section 2.2.6). After the cells had been washed, covered with fresh HBSS (37°C) and allowed to equilibrate for 45 min, they were incubated at 37°C with 500 nM isoproterenol for the indicated periods. Incubations were terminated by addition of ice-cold trichloroacetic acid (5% w/v). [^{3}H]cAMP was isolated from the acidified extracts (Section 2.2.8.) and expressed as pmol/mg of protein (Section 2.2.11.). Values are means ± S.E.M. from 3 determinations in the same experiment.



1000
800
600
400
200
0
0
1
2
3
[3H]cAMP (pmol/mg of protein)
Incubation period (min)

Fig. 5.5. SNP-induced increases in [3H]cGMP in cultured (W1) VSMC

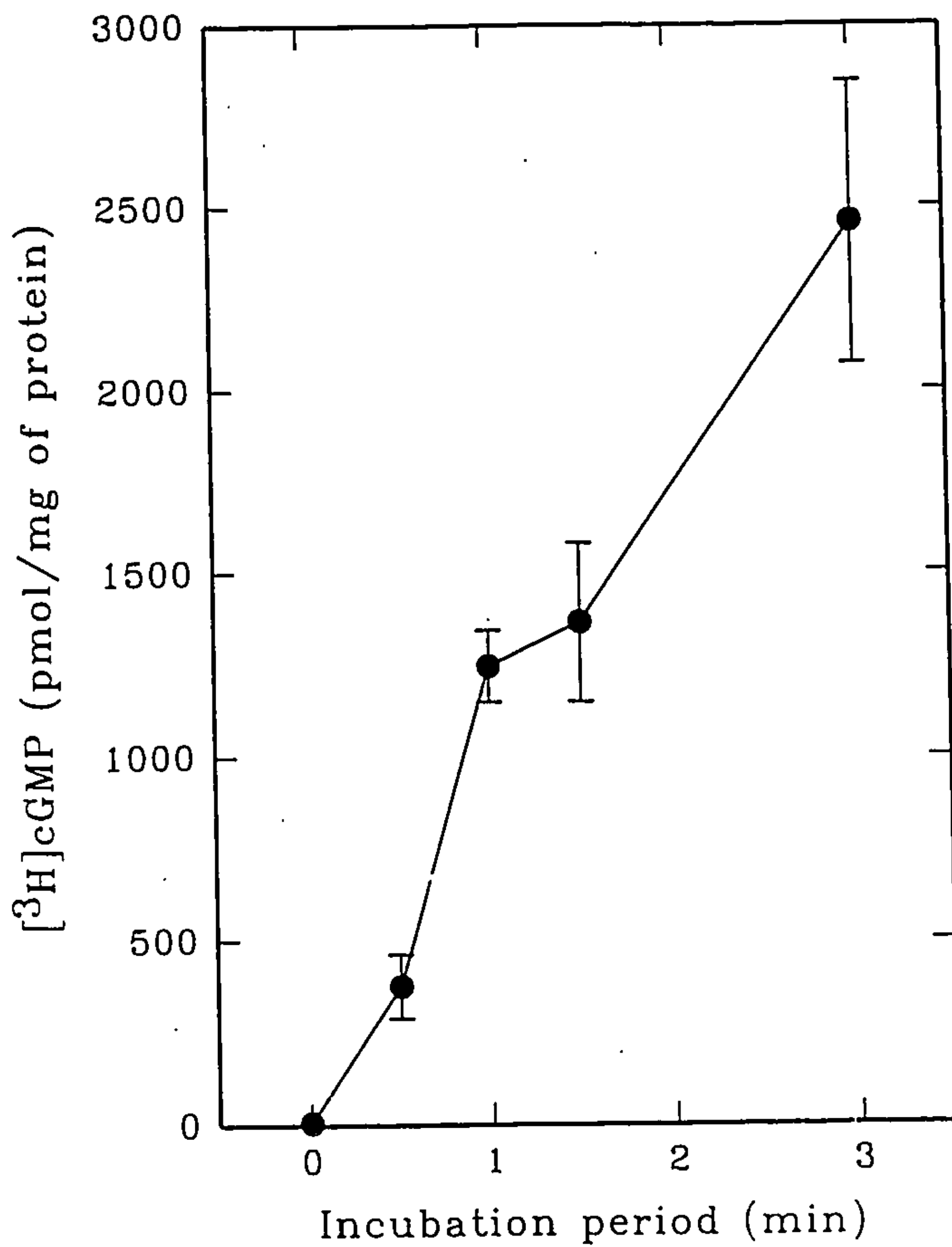
Confluent cultured (W2) VSMC were labelled with [^{3}H]hypoxanthine. After being washed and covered with fresh HBSS (37°C), the labelled cells were incubated with the indicated concentrations of SNP for 1 min at 37°C. Incubations were terminated by addition of ice-cold trichloroacetic acid (final concentration 5% (w/v)). Values are means ± S.E.M. from the cells in 3 separate wells.



1200
1000
800
600
400
200
0
[3H]cGMP (pmol/mg of protein)
∞
7
6
5
- log [SNP] (M)

Fig. 5.6. Time course of the increases in [3H]cGMP caused by SNP in (W1) VSMC

Confluent cultures of (W1) VSMC were labelled with [^{3}H]hypoxanthine overnight. The labelled cells were washed three times with fresh HBSS and then covered with 0.45 ml of fresh HBSS (37°C). The cells were incubated with SNP at 37°C for the indicated periods. Incubations were terminated by addition of ice-cold trichloroacetic acid (final concentration 5% (w/v)). Values are means ± S.E.M. from 3 determinations in the same experiment.



3000
2500
2000
1500
1000
500
0
[3H]cGMP (pmol/mg of protein)
0
1
2
3
Incubation period (min)

Fig. 5.7. Increases in [^{3}H]cAMP in cultured (W2) VSMC caused by adenosine

Confluent cultured VSMC (W2) were labelled overnight with [^{3}H]hypoxanthine and then washed and covered with 0.45 ml fresh HBSS (37°C). The cells were incubated with the indicated concentrations of adenosine for 1 min, following which they were extracted with ice-cold trichloroacetic acid (final concentration 5% (w/v)). Values are means $\pm$ S.E.M. from 3 determinations in the same experiment.

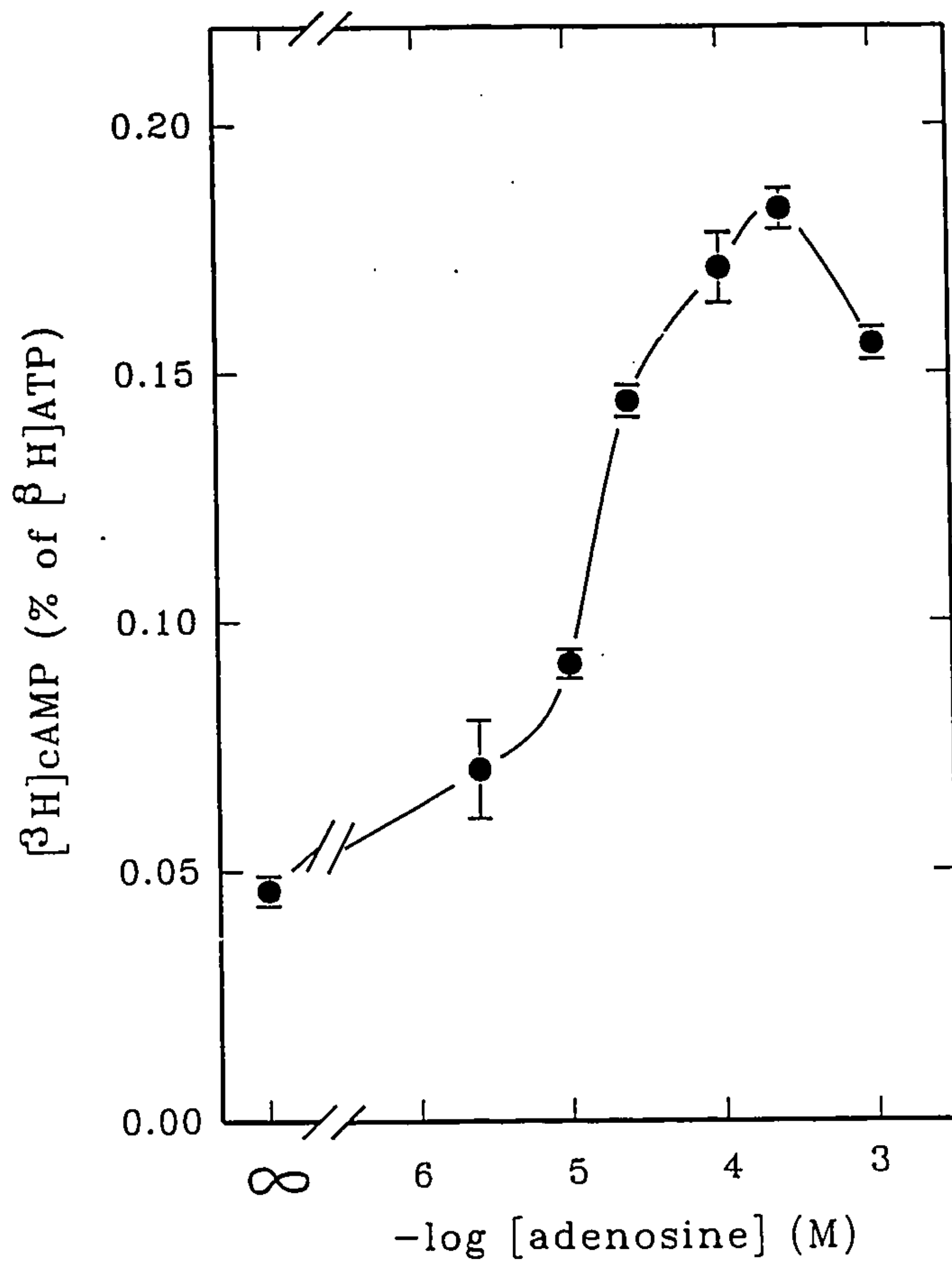
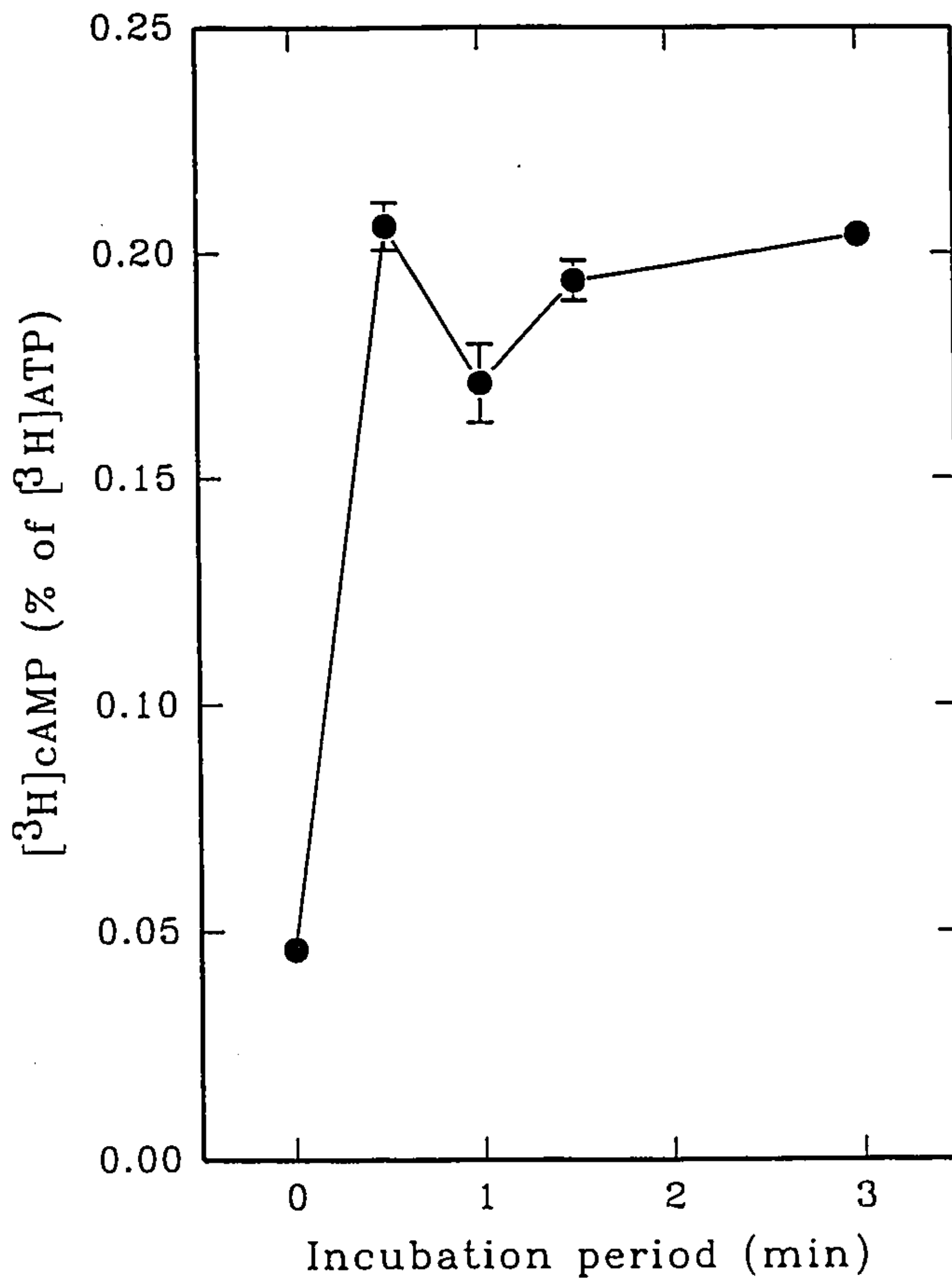

0.20
0.15
0.10
0.05
0.00
[3H]cAMP (% of [3H]ATP)
∞
6
5
4
3
-log [adenosine] (M)

Fig. 5.8. Time dependence of adenosine-induced increases in [^{3}H]cAMP in prelabelled cultured (W2) VSMC

Prelabelled VSMC were incubated with 100 μM adenosine for the indicated periods. The incubations were terminated by addition of ice-cold trichloroacetic acid (final concentration 5% (w/v)). [^{3}H]cAMP is expressed as a percentage of the total [^{3}H]ATP in each well. Values are means ± S.E.M. from 3 determinations in the same experiment.



0.25
0.20
0.15
0.10
0.05
0.00
[3H]cAMP (% of [3H]ATP)
0
1
2
3
Incubation period (min)

Table 5.1. Effects of isoproterenol or adenosine on the level of [^{3}H]cAMP in different cultured VSMC lines

VSMC were subcultured into 24 well plates and once confluent each well was labelled for 4 h with [^{3}H]adenine (2 μCi, 2 μM final concentration). Cells were washed two times with HBSS at 37°C and then covered with 0.45 ml of fresh HBSS. Cells were incubated with isoproterenol (Iso) or adenosine (AD) for 1 min at 37°C. Incubations were terminated by addition of 0.5 ml of 10% (w/v) ice-cold trichloroacetic acid. [^{14}C]cAMP (approx. 700 d.p.m.) was added to each well, the contents were transferred to numbered polypropylene tubes and the supernatants were isolated by centrifugation (2 min at 12 000 x g). Cyclic nucleotides are expressed as percentages of the total ^{3}H in each well and values are means ± S.E.M. from three determinations in the same experiment.

Additions	[^{3}H]cAMP (% of [^{3}H]ATP)				
	W1	W2	W3	W4	W5
None	0.02 ± 0.00	0.03 ± 0.00	0.03 ± 0.00	0.04 ± 0.00	0.02 ± 0.00
Iso (1.0 μM)	0.81 ± 0.02	0.03 ± 0.00	0.04 ± 0.00	0.04 ± 0.00	0.03 ± 0.00
Iso (10 μM)	1.06 ± 0.02	0.03 ± 0.00	0.04 ± 0.01	0.04 ± 0.00	0.02 ± 0.00
AD (1.0 μM)	0.02 ± 0.00	0.04 ± 0.00	0.07 ± 0.00	0.04 ± 0.00	0.03 ± 0.00
AD (10 μM)	0.02 ± 0.00	0.06 ± 0.01	0.12 ± 0.01	0.16 ± 0.02	0.06 ± 0.00

Table 5.2. Pharmacological characteristics of rat aortic cultured VSMC

Agent	Response	Cell line	
		W1	W2
Isoproterenol	increase in cAMP	YES	NO
Adenosine	increase in cAMP	NO	YES
SNP	increase in cGMP	YES	NO
SNP	increase in cAMP	NO	NO
Cilostamide	increase in cAMP	NO	NO
Ro 20,1724	increase in cAMP	YES	YES

Table 5.3. Purity of $[^3H]$cAMP and $[^3H]$cGMP isolated from prelabelled cultured VSMC by the two column method

Cultured VSMC that had been prelabelled with [3H]hypoxanthine were incubated with either isoproterenol (Iso) or SNP for 1 min. Incubations were terminated by addition of ice-cold trichloroacetic acid (final concentration 5% (w/v)). Both cyclic nucleotides were first isolated by chromatography on alumina and Dowex-50 and then further purified by t.l.c. (Section 2.2.9). Values are means ± S.E.M. from 3 identical incubation mixtures in the same experiment; significant differences between the ratios before and after t.l.c. are indicated; $^{*}p < 0.05$.

Additions	Labelled cyclic nucleotide	Ratio of $^3H/^{14}C$ before t.l.c.	Ratio of $^3H/^{14}C$ after t.l.c.
None	cGMP	0.63 ± 0.03	0.46 ± 0.01*
	cAMP	1.90 ± 0.04	1.65 ± 0.06*
Iso(10 μM)	cGMP	0.67 ± 0.06	0.46 ± 0.06
	cAMP	46.50 ± 4.25	42.30 ± 1.59
SNP (100 μM)	cGMP	7.08 ± 0.17	6.69 ± 0.39
	cAMP	1.83 ± 0.08	1.74 ± 0.26

Table 5.4. Comparison of VSMC cGMP and cAMP content as determined using prelabelling assays and radioimmunoassays

Cultured VSMC were grown in 24 well culture dishes until confluent. After replacement of the medium, the cells were incubated overnight with fresh DMEM containing either $[^3H]$hypoxanthine (10 Ci/mmol) or unlabelled hypoxanthine, at a final concentration of 2 μM. Cells incubated with $[^3H]$hypoxanthine were used to measure $[^3H]$cAMP and $[^3H]$cGMP by a prelabelling assay, while those incubated with unlabelled hypoxanthine were used to measure changes in cAMP and cGMP by radioimmunoassay. After they had been washed three times with HBSS (37°C), the cells were covered with 0.45 ml of fresh HBSS and placed in the incubator for 45 min. Each sample of cells was incubated for 1 min at 37°C with isoproterenol (Iso) or SNP as indicated. Incubations were terminated by addition of ice-cold trichloroacetic acid (final concentration 5% (w/v)). $[^{14}C]$cAMP and $[^{14}C]$cGMP (700 d.p.m. each) were added to monitor the recoveries of cyclic $[^3H]$nucleotides in the prelabelling assay, whereas 7000 d.p.m. of $[^3H]$cAMP and of $[^3H]$cGMP were added to the samples used in the radioimmunoassay. The cyclic $[^3H]$nucleotides from the prelabelling assay were separated and purified (Section 2.2.8.) and, using the specific activities of the $[^3H]$ATP and $[^3H]$GTP determined in control samples (Section 2.2.11.), expressed as pmol/mg of protein. Similarly, the cyclic nucleotides determined using the radioimmunoassay (Section 2.2.13.) were expressed in pmol/mg of protein. Values from the prelabelling assay are means $\pm$ S.E.M. from 3 determinations; each determination is based on the value obtained from one well of cells. Values from the radioimmunoassays are means $\pm$ S.E.M. from 3 determinations; each determination is based on the amount of cyclic nucleotide in cells from 4 wells. Significant differences between the two methods are shown; ($^*p < 0.05$).

Table 5.4.

Additions	cAMP (pmol/mg of protein)		cGMP (pmol/mg of protein)	
	Prelabelling assay	Radioimmunoassay	Prelabelling assay	Radioimmunoassay
None	6.9 ± 0.6	9.5 ± 0.6*	6.7 ± 0.6	6.0 ± 0.5
Iso (10 μM)	966.2 ± 29.7	1126.7 ± 99.6	6.6 ± 0.4	5.5 ± 0.5
SNP (10 μM)	8.2 ± 1.1	9.0 ± 0.8	2349.1 ± 151.2	2089.9 ± 292.8
Iso (10 μM) + SNP (10 μM)	1114.4 ± 115.3	1236.1 ± 38.0	2408.0 ± 72.5	1857.6 ± 176.7

PAGINATION ERROR.

ERREUR DE PAGINATION.

TEXT COMPLETE.

LE TEXTE EST COMPLET.

NATIONAL LIBRARY OF CANADA.

CANADIAN THESES SERVICE.

BIBLIOTHEQUE NATIONALE DU CANADA.

SERVICE DES THESES CANADIENNES.

Table 5.5. Effects of different vasodilators on the levels of $[^3H]$cAMP and $[^3H]$cGMP in two distinct cultured VSMC lines

Cultured VSMC were grown in 24 well dishes until confluent and then labelled overnight with $[^3H]$hypoxanthine (10 Ci/mmol; 2 μM). Following removal of the labelling medium, the cells were washed with fresh HBSS and then 0.45 ml of fresh HBSS was added to each well. Cells were incubated with the vasodilators (isoproterenol (Iso), adenosine (AD) and SNP) either singly or in combination (as indicated) for 1 min at 37°C. Incubations were terminated by addition of ice-cold trichloroacetic acid (final concentration 5 % (w/v)). $[^3H]$cAMP and $[^3H]$cGMP isolated from each sample are expressed as a percentage of the corresponding $[^3H]$nucleoside triphosphate. Values are means ± S.E.M. from 8 (W1) or 3 (W2) experiments. Significant changes in cyclic $[^3H]$nucleotide levels are indicated; $^{*}p < 0.05$, $^{**}p < 0.001$.

VSMC line	Additions	$[^3H]$cAMP (% of $[^3H]$ATP)	$[^3H]$cGMP (% of $[^3H]$GTP)
W1	None	0.08 ± 0.01	0.07 ± 0.01
	Iso (1.0 μM)	3.32 ± 0.48**	0.08 ± 0.01
	SNP (10 μM)	0.09 ± 0.02	5.85 ± 2.55**
	Iso (1.0 μM) + SNP (10 μM)	3.10 ± 0.46**	4.93 ± 1.68**
W2	None	0.08 ± 0.02	0.04 ± 0.02
	AD (100 μM)	0.30 ± 0.05*	0.04 ± 0.01
	SNP (100 μM)	0.09 ± 0.02	0.03 ± 0.01
	AD (100 μM) + SNP (100 μM)	0.29 ± 0.03*	0.03 ± 0.01

Table 5.6. Effects of SNP, cilostamide and Ro 20-1724 on the increases in [^{3}H]cAMP caused by isoproterenol in cultured (W1) VSMC

Cultured VSMC (W1) that had been prelabelled with [3H]hypoxanthine were incubated for 1 min at 37° with the indicated concentrations of the following compounds; Iso, isoproterenol; SNP; Cil, cilostamide; Ro, Ro 20-1724. Incubations were terminated by addition of trichloroacetic acid (5% w/v) and the [^{3}H]cAMP isolated from the acidified extracts. Values are means ± S.E.M. from 3 determinations in the same experiment and those significantly different from the controls are indicated; $^*p < 0.001$; $^{**}p < 0.001$. Also, a significant effect of Ro 20-1724 is noted; ^{a} $p < 0.001$.

Additions	[^{3}H]cAMP (% of [^{3}H]ATP)	[^{3}H]cGMP (% of [^{3}H]GTP)
None	0.06 ± 0.00	0.08 ± 0.01
Iso (0.1 µM)	2.10 ± 0.03**	0.07 ± 0.01
SNP (10 µM)	0.05 ± 0.00	15.00 ± 1.00**
Cil (10 µM)	0.06 ± 0.00	0.06 ± 0.01
Ro (300 µM)	0.11 ± 0.01*	0.07 ± 0.01
Iso (0.1 µM) + SNP (10 µM)	2.17 ± 0.00**	14.50 ± 2.11**
Iso (0.1 µM) + Cil (10 µM)	2.17 ± 0.05**	0.07 ± 0.01
Iso (0.1 µM) + Ro (300 µM)	4.39 ± 0.13**a	0.08 ± 0.01

Table 5.7. Effects of SNP, cilostamide and Ro 20-1724 on the increases in $[^3H]$cAMP induced by adenosine in cultured (W2) VSMC

Confluent cultures of (W2) VSMC were labelled overnight with $[^3H]$hypoxanthine. After they had been washed and then covered with fresh HBSS (37°C), the cells were incubated with the indicated compounds for 1 min at 37°C (AD, adenosine; SNP; Cil, cilostamide; Ro, Ro 20-1724). Incubations were terminated by the addition of ice-cold trichloroacetic acid (final concentration 5 % (w/v)). Values are means ± S.E.M. from 3 determinations from the same experiment and those values significantly different from the controls are indicated; $^*p < 0.05$ and $^{**}p < 0.001$.

Additions	$[^3H]$cAMP (% of $[^3H]$ATP)	$[^3H]$cGMP (% of $[^3H]$GTP)
None	0.04 ± 0.00	0.08 ± 0.03
AD (100 μM)	0.08 ± 0.01*	0.05 ± 0.01
SNP (100 μM)	0.04 ± 0.00	0.04 ± 0.01
Cil (10 μM)	0.05 ± 0.00	0.04 ± 0.00
Ro (300 μM)	0.08 ± 0.01*	0.06 ± 0.01
AD (100 μM) + SNP (100 μM)	0.10 ± 0.03	0.03 ± 0.00
AD (100 μM) + Cil (10 μM)	0.11 ± 0.02	0.04 ± 0.01
AD (100 μM) + Ro (300 μM)	4.92 ± 0.13**	0.06 ± 0.01

Chapter 6. *General Discussion*

6.1. *General discussion*

6.1.1. *Novel findings reported in this thesis*

I have carried out experiments that led to the elucidation of the molecular basis of the synergistic inhibition of platelet function by nitrovasodilators and activators of adenylyl cyclase (Chapter 3). This work is the first to demonstrate a nitrovasodilator-induced potentiation of the increases in platelet cAMP caused by activation of adenylyl cyclase. In addition, these results demonstrate that cAMP, not cGMP, is the cyclic nucleotide involved in the synergistic inhibition of platelet function caused by simultaneous activation of adenylyl and guanylyl cyclases. Although potentiation of the effects of PGI_2 by SNP (Levin *et al.*, 1982) or EDRF (Radomski *et al.*, 1987; Macdonald *et al.*, 1988) had previously been described, the effects of SIN-1 on the PGE_1-induced inhibition of platelet function and the reported synergism between SNP or SIN-1 and adenosine are novel. Also, this study is the first to demonstrate that cGMP can exert cellular effects by inhibiting the cGI-PDE.

I have also demonstrated a synergistic effect of nitrovasodilators and isoproterenol on VSM tone. To my knowledge, this is the first study describing a nitrovasodilator-induced potentiation of the effects of isoproterenol (or any other activator of VSMC adenylyl cyclase) on either relaxation or inhibition of contraction of VSM. In addition, the

state of the VSM (i.e. contracted vs non-contracted) was shown to be an important consideration in the study of synergism between agents that act by increasing cyclic nucleotides. The potentiation by SNP of the isoproterenol-induced increase in VSM cAMP and the increase in cAMP caused by a higher concentration of the nitrovasodilator used alone, are also the first significant effects of a nitrovasodilator on VSM cAMP to be documented. Moreover, the correlation between these increases in cAMP and the effects of SNP on VSM tone suggest that cAMP may play a role in the effects of this agent on VSMC function. Although an earlier report demonstrated that the PGI_2-induced relaxation of porcine arteries could be potentiated by EDRF present in an endothelial superfusate (Shimokawa *et al.*, 1988), the authors did not measure the levels of either cyclic nucleotide in this tissue, nor did they postulate a mechanism to explain the effects they observed.

To date, most investigators interested in the effects of cAMP or cGMP in platelets or VSMC function, have viewed the two cyclic nucleotides as messengers of independent parallel signal transduction systems. Thus, when platelet or VSM responses were inhibited by incubation with compounds known to activate either adenylyl or guanylyl cyclases, the effects were said to be mediated by cAMP or cGMP, respectively. Indeed, in several reports (Tateson *et al.*, 1977; Kukovetz *et al.*, 1979; Nishikawa *et al.*, 1982; Silver *et al.*, 1982), strong correlations between the drug-induced changes in cAMP *or* cGMP levels and drug-induced inhibition of function have

been reported. Also, two recent reviews demonstrate that many believe that cAMP and cGMP act independently of one another. In one, Lincoln (1989) discusses the role(s) of cGMP in drug- or hormone-induced smooth muscle relaxation and it is clear that the author views the effects of cGMP in VSM function as independent of any effect attributable to the activation of the adenylyl cyclase/cAMP system. This dissociation of the roles played by the two cyclic nucleotides is evidenced by the statement that "it is cGMP (not cAMP) which may be the more important regulator of smooth muscle relaxation". Similarly, in a review of the mechanism(s) of action of cAMP in drug- or hormone-induced smooth muscle relaxation, Murray (1990) describes the relaxation caused by agents that activate adenylyl cyclase in the context of cAMP-mediated effects with little or no mention of the importance of cGMP, even though the author acknowledges the presence of a cGI-PDE in both platelets and VSM. However, in a more recent publication, Lincoln *et al.* (1990) proposes that some of the effects of cAMP may actually be mediated by activation of the cGMP-PK in cultured VSMC from rat aorta. Also, in a review of the effects of cGMP on platelet and VSM function, Walter (1989), proposes that interactions between the two cyclic nucleotides could occur.

6.1.2. *Role(s) of cyclic nucleotides in mediating inhibitory effects*

As described in Chapters 3 and 4 of this thesis, previous attempts

to measure increases in cAMP following incubation of platelets or VSM with nitrovasodilators (including EDRF) have for the most part been unsuccessful. I believe the reason for the majority of failures to be methodological. The inaccuracy of the methodological approaches used in most of these studies has been such that only very large increases in cAMP would have been detected (e.g Halbrügge *et al.*, 1990). The role of cGI-PDE in mediating the effects of cGMP in platelet and VSM function described here is complementary to an opposite role for a cGS-PDE in other cells. Thus, it has been postulated that a cGS-PDE is responsible for the agonist-induced reductions in the levels of cAMP in certain cells (Fischmeister and Hartzell, 1990; MacFarland *et al.*, 1991). Although small amounts of a cGS-PDE are known to be present in platelets (Grant *et al.*, 1990), the results described in this thesis demonstrate that the net effect of an increase in platelet cGMP is inhibition of cAMP breakdown, not stimulation.

Although the supra-additive accumulations of cAMP in both platelets and VSM described in this thesis correlate very well with the synergistic inhibitions of function in both tissues, the mechanism(s) by which this cyclic nucleotide mediates these inhibitions is not clear. The most probable mechanism involves the activation of cAMP-PK and the subsequent phosphorylation of specific protein substrates. cAMP-PK activity was not measured in the present study but, since only modest increases in cAMP are required for activation of the cAMP-PK (Halbrügge *et al.*, 1990), cAMP-mediated phosphorylations probably mediate

most of these inhibitory effects. The small increase in cAMP required for activation of the cAMP-PK may reflect the fact that a large percentage of the cAMP present in resting cells is bound to the regulatory subunit of inactive cAMP-PK (Khac *et al.*, 1973). Since the binding of cAMP to the two sites of the regulatory subunit of this kinase has positivly cooperative effects on cAMP-PK activation (see Section 1.11.), the presence of cAMP bound to the inactive enzyme reduces the percentage increase in cAMP required for full activation. The inhibition of VSM contraction by 100 nM isoproterenol, in the absence of easily measurable increases in cAMP (see Chapter 4), is consistent with this, though cAMP-independent actions of isoproterenol cannot be ruled out. However, cAMP also has effects on the cGMP-PK (see Section 1.13.2.). Thus, cAMP-stimulated autophosphorylation of purified cGMP-PK has been shown to decrease the concentration of cAMP required for activation of this kinase and also to decrease the rate of dissociation of cGMP from Site 1 (see Section 1.13.2.). However, since most of these effects have been studied *in vitro* with purified cGMP-PK, the physiological importance of cAMP-mediated cGMP-PK activation is not clear. If such an interaction were possible *in vivo*, a simultaneous increase in cAMP and cGMP could have marked effects on cGMP-PK activity. In this context, Lincoln *et al.* (1990) has recently suggested that the activation of VSMC cGMP-PK by cAMP mediates the forskolin- and isoproterenol-induced inhibition of the [8-arginine]vasopressin-induced Ca^{2+} transients in cultured rat aortic VSMC.

6.1.3. *Physiological significance of synergism between nitrovasodilators and activators of adenylyl cyclase in platelets and VSMC*

The synergistic effects of activators of adenylyl and guanylyl cyclases described in this thesis may have broad physiological significance. Thus, the EDRF synthesized from L-arginine by the vascular endothelium (Palmer *et al.*, 1987) and PGI_2, also produced by the vascular endothelium (Moncada and Vane, 1979), have been shown to inhibit platelet aggregation synergistically *in vitro* (Radomski *et al.*, 1987; Macdonald *et al.*, 1988). The release of these agents may also have synergistic inhibitory effects on platelet aggregation *in vivo*. Thus, Bhardwaj *et al.* (1988) and Bertolini *et al.* (1990) have both shown that EDRF inhibits platelet aggregation in whole blood and Bowen and Haslam (1991) have shown that SIN-1 and PGE_1 act synergistically to inhibit platelet function in whole blood. Again, the latter effect was shown to be mediated by a supra-additive increase in platelet cAMP (Bowen and Haslam, 1991). These findings are particularly significant in light of the fact that hemoglobin traps nitric oxide and is a potent inhibitor of the effects of both nitrovasodilators and EDRF. Results of *in vivo* experiments are fewer and more difficult to interpret. Bhardwaj *et al.* (1988) also reported the *in vivo* inhibition of platelet aggregation by EDRF and Hogan *et al.* (1988) found that a bolus injection of carbachol into anaesthetized rabbits resulted in inhibition of platelet aggregation and a significant

increase in the level of platelet cGMP. In the latter study, both effects were attributed to a carbachol-induced release of EDRF from the vascular endothelium. However, cAMP levels were not measured in these experiments. Thus, it remains to be determined if PGI_2 and EDRF cause synergistic inhibition of platelet function *in vivo* and whether this effect is associated with increased accumulation of cAMP. Also, since EDRF is continuously released by endothelium (Martin *et al.*, 1986), this factor may contribute to the resting level of cGMP in circulating platelets. Thus, platelets in the circulation may be poised to respond optimally to activators of adenylyl cyclase, such as PGI_2 or adenosine. In this context, it is of interest that the basal level of cGMP determined by Hogan *et al.* (1988) was substantially higher than that reported in this thesis for washed platelets. Also, platelets have recently been shown to be capable of synthesising nitric oxide (Radomski *et al.*, 1990a). This nitric oxide formation has been shown to account for the formation of cGMP during platelet aggregation. Thus, small aggregating stimuli may allow the formation of cGMP and this cGMP may synergize with activators of adenylyl cyclase to inhibit the aggregation. This system would allow for very potent feedback inhibition.

This thesis also documents synergistic interactions between activators of adenylyl and guanylyl cyclases in VSM (Chapter 4). If such an interaction occured *in vivo*, potent vasorelaxant effects could arise as a consequence of the simultaneous actions of PGI_2 and

EDRF on VSM. Although individually PGI_2 and EDRF are well known to be potent vasorelaxant agents *in vitro* and *in vivo*, virtually nothing is known about the combined effects of these agents on VSMC function. In man, infusion of PGI_2 causes marked vasorelaxation and increases heart rate (Moncada and Vane, 1979). However, this compound is not a circulating hormone (e.g. Haslam and McClenaghan, 1981). The physiological role of EDRF has only recently been assessed. Collier and Vallance (1989) demonstrated that removal of the vascular endothelium of the large veins of the back of the hand, led to a marked vasoconstriction of these veins and a reduced vasodilation in response to acetylcholine. Recently, Gardiner *et al.* (1990) have shown that N^G-monomethyl-L-arginine (L-NMMA), an inhibitor of nitric oxide synthase, caused dose-dependent increases in blood pressure and bradycardia in the rat. Thus, L-NMMA caused marked vasoconstriction of the carotid, mesenteric, renal and hindquarter vascular beds (Gardiner *et al.*, 1990).

Nitric oxide can also be produced and released by VSMC (Wood *et al.*, 1989). Also, this VSM-derived nitric oxide has recently been shown to have important functional effects on vascular tone (Schini and Vanhoutte, 1990). In this study, incubation of de-endothelialized rat aorta with L-arginine was shown to have marked effects on vascular tone. Thus, the effects obtained with nitric oxide synthesis inhibitors *in vivo* will have to be re-assessed in view of evidence that they can act on both vascular endothelium and VSMC. To date, the physiological relevance of the combined actions of EDRF and PGI_2 has

not been investigated. However, since inhibitors of the cGI-PDE have been shown to potentiate the effects of activators of adenylyl cyclase is many vascular beds (see Chapter 4), a synergistic interaction between these two compounds is to be expected. In one study, Willis *et al.*, (1989), reported that nitroglycerin did *not* potentiate the effects of a PGI_2 analogue in rats. Although the choice of animal (SHR) in these experiments may have contributed to the results, it may be important that reductions in basal tone rather than antagonism of an agonist-induced increase in tone were studied. In my experiments, synergism was much *less* evident when relaxation of VSM was studied. Also, endogenous EDRF may have already potentiated the effects of the PGI_2 analogue rendering further synergism less conspicuous. EDRF may also potentiate the effects of other endogenous activators of adenylyl cyclase. Thus, the calcitonin gene-related peptide (CGRP) has been shown to increase cAMP in rat aortic smooth muscle (Kubota *et al.*, 1985). Other endogenous activators of guanylyl cyclase may also potentiate the effects of activators of adenylyl cyclase. Thus, ANP (Hamet *et al.*, 1984), brain natriuretic peptide (BNP) (Sudoh *et al.*, 1988) and C-type natriuretic peptide (CNP) (Sudoh *et al.*, 1990) all activate particulate guanylyl cyclase in various tissues (Furuya *et al.*, 1990).

6.1.4. *Therapeutic implications of synergism*

A combination of specific agents that activate both adenylyl and

guanylyl cyclases only in the tissue of interest should allow selective effects to be achieved. Thus, it is of interest that the PGI_2 analogue (5Z)-carbacyclin preferentially acts on platelet PGI_2 receptors (Corsini *et al.*, 1987). This structural analogue of PGI_2 causes very little relaxation of rabbit mesenteric artery and also causes much smaller increases in mesenteric artery cAMP than either PGE_1 or PGI_2 (Corsini *et al.*, 1987). In the simultaneous presence of a low concentration of a nitrovasodilator with non-specific effects, this or other selective activators of adenylyl cyclases might be expected to have particularly potent antithrombotic effects. Another possible approach by which tissue selective effects could be achieved has been considered by Bowen and Haslam (1991). These authors suggested that a compound that could simultaneously increase cAMP and cGMP could have particularly potent effects. Thus, dipyridamole which inhibits platelet cGMP-PDE (Weishaar *et al.*, 1986) and blocks adenosine uptake by blood cells, so causing an adenosine and cAMP-mediated inhibition of platelet aggregation (Gresele *et al.*, 1983), could have potent effects on platelet function *in vivo*. Whereas platelets contain only soluble guanylyl cyclase, VSMC contain both soluble and membrane-bound guanylyl cyclase activities. Thus, tissue-specific functional synergism could be achieved in VSM by activation of the particulate guanylyl cyclase, rather than the soluble enzyme, by using ANP analogues. Thus, in principle, selective synergistic effects of therapeutic interest might be obtained through the use of combinations of pharmacological agents, each of limited specificity, that together

activate both adenylyl cyclase and guanylyl cyclase only in the target tissue.

6.1.5. *cGI-PDE inhibition in other tissues*

Although the presence of cGI-PDE has been reported in platelets, VSM, cardiac muscle and adipose tissue, effects of cGMP-elevating agents on cAMP levels in intact tissues have not been demonstrated in either cardiac muscle or adipose tissue. Thus, it is difficult to assess the importance of a cGMP-mediated inhibition of this enzyme in regulating the effects of agents that activate adenylyl cyclase in these tissues. In guinea pig atria, 8BrcGMP significantly reduced the positive inotropic effects of a series of cGI-PDE inhibitors (Muller *et al.*, 1990). Although a cGS-PDE has been suggested to mediate this effect (Fischmeister and Hartzell, 1990), 8BrcGMP is a poor activator of this enzyme (Erneux *et al.*, 1985). The effects of cGMP, or its analogues, as cGI-PDE inhibitors in adipose tissue have not been studied.

Canine tracheal smooth muscle was recently shown to contain a cGI-PDE (Torphy *et al.*, 1990). The enzyme isolated from this tissue was very similar to the cardiac and platelet enzymes, both with respect to its inhibition by SK&F 94120 and its kinetic characteristics. In an earlier study, Torphy *et al.* (1985) had demonstrated that SNP caused a significant increase in the level of cAMP in canine tracheal smooth

muscle. This effect was blocked by indomethacin and, for this reason, later work was carried out in the presence of this cyclooxygenase inhibitor (Torphy *et al.*, 1985). Thus, the possibility exists that the SNP-induced increase in cAMP in this tissue represents potentiation of a prostaglandin-stimulated accumulation of cAMP, as a result of an effect of cGMP on the tracheal smooth muscle cGI-PDE. However, the effects of simultaneous addition of agents that activate adenylyl and guanylyl cyclases in this tissue have not been investigated. If a synergistic increase in cAMP results from such a treatment, a combination of a β-adrenergic agonist and a vaporizable nitrovasodilator might be beneficial in the treatment of bronchial conditions, such as asthma.

As described in Section 6.1.3., endothelial cells are not the only cells capable of generating nitric oxide from L-arginine. One of the first cell types shown to have this capacity was the macrophage (Hibbs *et al.*, 1987a). The generation of nitric oxide from L-arginine in macrophages has been shown to be involved in the cytotoxicity of these cells (Hibbs *et al.*, 1987b). However, the system by which nitric oxide generation in macrophages may be regulated appears to be markedly different from that in endothelial cells. Thus, it has been shown that the release of macrophage-derived nitric oxide occurs only several hours after stimulation of the cells (Marletta *et al.*, 1988). Also, increases in $[Ca^{2+}{}_{i}]$ may not be required for nitric oxide formation in macrophages (Hauschildt *et al.*, 1990). However, since these

cells generate nitric oxide they represent a cell type in which the presence of a cGI-PDE would allow synergistic interactions between endogenous nitric oxide-mediated formation of cGMP and activators of adenylyl cyclase. The different types of PDEs present in macrophages have, however, not yet been studied. The synthesis of nitric oxide has also been shown in neutrophils (McCall *et al.*, 1989). The formation of this neutrophil-derived nitric oxide was shown to be inhibited by L-NMMA. Also, incubation of neutrophils with platelets inhibited platelet aggregation (McCall *et al.*, 1989), and incubation of these cells with smooth muscle caused relaxation (Rimele *et al.*, 1988). Thus, since neutrophils synthesize nitric oxide, the presence of a cGI-PDE could bring about marked effects when these cells are stimulated. Also, since the neutrophil-derived nitric oxide is released continuously (McCall *et al.*, 1989), its presence might make neutrophils very susceptible to agonists that activate adenylyl cyclase. A case in point might be the effects of adenosine on polymorphonuclear cells described by Neilson and Vestal (1989). In these experiments, adenosine increased cAMP and inhibited neutrophil function at concentrations as low as 1 nM. Based on studies with cAMP PDE inhibitors, Wright *et al.* (1990) have reported that a cGMP-insensitive PDE is a more important PDE in the neutrophil. However, in one report, the cGI-PDE inhibitor milrinone was shown to suppress neutrophil function and to synergize with PGE_1 (Verghese and Frangakis, 1988). In this context, Arnold *et al.* (1977) reported that SNP not only increased cGMP in polymorphonuclear leukocytes but

also caused significant increases in the levels of cAMP. Although these results were never published in full, Arnold *et al.* (1977) report in the discussion of their paper that this compound increased cAMP levels by as much as 5- to 20-fold in these cells. Thus, nitrovasodilator-induced increases in cAMP in these cells may represent an effect of cGMP on a neutrophil cGI-PDE. Also, since the formation of nitric oxide in these cells is dependent on an increase in $[Ca^{2+}{}_{i}]$, synergistic increases in cAMP might result from simultaneous incubation of these cells with chemoattractants that stimulate Ca^{2+} entry and activators of adenylyl cyclase. Thus, it is perhaps significant that a synergistic increase in cAMP was reported in neutrophils incubated with a Ca^{2+} ionophore and adenosine (Neilson and Vestal, 1989). However, cGMP levels were not measured in this study.

Effects mediated by nitric oxide have also been reported in nervous tissue. Although the initial report was related to CNS effects mediated by activation of N-methyl-D-aspartate (NMDA)/glutamate receptors (Garthwaite *et al.*, 1989), several indications that peripheral neurons may also be able to release nitric oxide have been published recently (Gillespie *et al.*, 1989; Gibson *et al.*, 1990; Ignarro *et al.*, 1990). In cerebellum, the formation and release of nitric oxide was shown to be coupled to activation of the NMDA receptor and to occur as a result of a agonist-induced influx of Ca^{2+} (Garthwaite *et al.*, 1989). In the case of peripheral autonomic neurons, nitric oxide has been proposed to mediate the non-adrenergic non-cholinergic

relaxation of anococcygeus muscle (Gibson *et al.*, 1990) and of corpus cavernosum smooth muscle (Ignarro *et al.*, 1990). To date, the presence of a cGI-PDE in these tissues has not been investigated, but synergistic interactions with neurotransmitters acting via a cAMP mechanism are conceivable.

Given the potential physiological and pharmacological importance of a general mechanism by which cGMP may potentiate the effects of cAMP, I believe that a major effort to determine whether synergistic increases in cAMP occur in the tissues and cells mentioned above is warranted.

Chapter 7. *References*

Abe, A. and H. Karaki, 1989, Effects of forskolin on cytosolic Ca^{2+} level and contraction in vascular smooth muscle. J. Pharmacol. Exp. Ther. 249:895-900

Adam, L.P., J.R. Haeberle and D.R. Hathaway, 1989, Phosphorylation of caldesmon in arterial smooth muscle. J. Biol. Chem. 264: 7698-7703

Adams, A.F. and R.J. Haslam, 1978, Factors affecting the activity of guanylate cyclase in lysates of human blood platelets. Biochem. J. 174:23-35

Adelstein, R.S. and M.A. Conti, 1975, Phosphorylation of platelet myosin increases actin-activated myosin ATPase activity. Nature 256:597-598

Adelstein, R.S., M.A. Conti, D.R. Hathaway and C.B. Klee, 1978, Phosphorylation of smooth muscle myosin light chain kinase by the catalytic subunit of adenosine 3':5'-monophosphate-dependent protein kinase. J. Biol. Chem. 253:8347-8350

Adunyah, S.E. and W.L. Dean, 1987, Regulation of human platelet membrane Ca^{2+} transport by cAMP- and calmodulin-dependent phosphorylation. Biochim. Biophys. Acta 930:401-409

Ahn, H.S., D. Eardley, R. Watkins and N. Prioli, 1986, Effects of several newer cardiotonic drugs on cardiac cyclic AMP metabolism. Biochem. Pharmacol. 35:1113-1121

Ahn, H.S., W. Crim., M. Romano, E. Sybertz and B. Pitts, 1989, Effects of selective inhibitors on cyclic nucleotide phosphodiesterases of rabbit aorta. Biochem. Pharmacol. 38:3331-3339

Aitken, A., B.A. Hemmings and F. Hofmann, 1984, Identification of the residues on cyclic GMP-dependent protein kinase that are autophosphorylated in the presence of cyclic AMP and cyclic GMP. Biochem. Biophys. Acta. 790:219-225

Akkerman, J.W.N., R.H.M. Ebberink, J.P.M. Lips, and G.C.M.L. Christiaens, 1980, Rapid separation of cytosol and particle fraction of human platelets by digitonin-induced cell damage. Br. J. Haematol. 44:291-300

Alheid, U., I. Reichwehr and U. Fostermann, 1989, Human endothelial cells inhibit platelet aggregation by separately stimulating platelet cyclic AMP and cyclic GMP. Eur. J. Pharmacol. 164:103-110

Anand-Srivastava, M., D.J. Franks, M. Cantin and J. Genest, 1982, Presence of Ra and P-site receptors for adenosine coupled to adenylate cyclase in cultured vascular smooth muscle cells. Biochem. Biophys. Res. Commun. 108:213-219

Angus, J.A. and T.M. Cocks, 1989, Endothelium-derived relaxing factor. Pharmac. Ther. 41:303-352

Antunes, E., P.S. Lidbury, G. de Nucci, and J.R. Vane, 1988, Lack of synergism of iloprost and sodium nitroprusside on rabbit vascular smooth muscle. Br. J. Pharmacol. 95:516P

Appleman, M.M. and W.L. Terasaki, 1975, Regulation of cyclic nucleotide phosphodiesterase. Adv. Cycl. Nucl. Res. 5:153-162

Ardlie, N.G., M.A. Packham, and J.F. Mustard, 1970, Adenosine diphosphate-induced platelet aggregation in suspensions of washed rabbit platelets. Br. J. Haematol. 19:7-17

Arnold, W.P., C.K. Mittal, S. Katsuki and F. Murad, 1977, Nitric oxide activates guanylate cyclase and increases guanosine 3':5'-cyclic monophosphate levels in various tissue preparations. Proc. Natl. Acad. Sci. U.S.A. 74:3203-3207

Ashby, B., 1988, Cyclic AMP turnover in response to prostaglandins in intact platelets: Evidence for separate stimulatory and inhibitory prostaglandin receptors.. Sec. Mess. Phosphoproteins. 12:45-57

Ashby, B., 1989a, Model of prostaglandin-regulated cyclic AMP metabolism in intact platelets: Examination of time-dependent effects on adenylate cyclase and phosphodiesterase activities. Mol. Pharmacol. 36:866-873

Ashby, B., 1989b, Effects of thromboxane antagonists on prostaglandin regulation of platelet adenylate cyclase. Sec. Mess. Phosphoproteins. 12:241-250

Authi, K.S., B.J. Evenden and N. Crawford, 1986, Metabolic and functional consequences of introducing inositol 1,4,5-trisphosphate into saponin-permeabilized human platelets. Biochem. J. 233:709-718

Axelsson, K.L., R.G.G. Andersson and J.E.S. Wikberg, 1982, Vascular smooth muscle relaxation by nitro compounds: reduced relaxation and cGMP elevation in tolerant vessels and reversal of tolerance by dithiothreitol. Acta Pharmacol. Toxicol. 50:350-357

Azuma, H., M. Ishikawa and S. Sekizaki, 1986, Endothelium-dependent inhibition of platelet aggregation. Br. J. Pharmacol. 88:411-415

Baltenserger, K., E. Carafoli and M. Chiesi, 1988, The Ca^{2+}-pumping ATPase and the major substrates of the cGMP-dependent protein kinase in smooth muscle sarcolemma are distinct entities. Eur. J. Biochem. 172:7-16

Barrington, W.W., K.A. Jacobson, A.S. Hutchison, M. Williams and G.L. Stiles, 1989, Identification of the A_2 adenosine receptor binding subunit by photoaffinity crosslinking, Proc. Natl. Acad. Sci. U.S.A. 86:6572-6576

Basista, M., L. Grodzinska, and J. Swies, 1985, The influence of molsidomine and its active metabolite SIN-1 on fibrinolysis and platelet aggregation. Thromb. Haemost. 54:746-749

Baumgartner, H.R. and R. Muggli, 1976, Adhesion and aggregation: Morphological demonstration and quantitation in vivo and in vitro, in "Platelets in Biology and Pathology" Ed. Gordon, J.L. pp:23-60, Elsevier/North-Holland, Amsterdam

Bean, P., 1989, Classes of calcium channels in vertebrate cells. Ann. Rev. Physiol. 51:367-384

Beavo, J.A., 1988, Multiple isozymes of cyclic nucleotide phosphodiesterase. Adv. Sec. Mess. Phosphoprotein Res. 22:1-38

Beavo, J., 1990, Multiple phosphodiesterase isozymes, background, nomenclature and implications, in "Cyclic Nucleotide Phosphodiesterases: Structure, Regulation and Drug Action", Eds. Beavo, J. and M.D. Houslay, pp.3-15, Wiley, Chichester

Beavo, J.A., J.G. Hardman and E.W. Sutherland, 1971, Stimulation of adenosine 3',5'-monophosphate hydrolysis by guanosine 3',5'-monophosphate. J. Biol. Chem. 246:3841-3846

Beavo, J.A. and D.H. Reifsnyder, 1990, Primary sequence of cyclic nucleotide phosphodiesterase isozymes and the design of selective inhibitors. Trends Pharmacol. Sci. 11:150-155

Beebe, S.J. and J.D. Corbin, 1986, Cyclic nucleotide-dependent protein kinases, in "The Enzymes", Eds. Boyer, P.D. and E.G. Krebs, 17:43-111, Academic Press, Orlando.

Benham, C.D., T.B. Bolton, N.G. Byrne and W.A. Large, 1987, Action of externally applied adenosine triphosphate of single smooth muscle cells dispersed from rabbit ear artery. J. Physiol. 387:473-488

Benham, C.D. and R.W. Tsien, 1987, A novel receptor-operated Ca^{2+}-permeable channel activated by ATP in smooth muscle. Nature 328:275-278

Benham, C.D. and R.W. Tsien, 1988, Noradrenaline modulation of calcium channels in single smooth muscle cells from rabbit ear artery. J. Physiol. 404:767-784

Bertolino, G., M. Vigotti, P. Noris, G.C. Piletta, F. Piovella and C.L. Balduini, 1990, Effect of endothelial cells on platelet aggregation in platelet rich plasma (PRP) and whole blood. Thromb. Res. 59:407-412

Berridge, M.J., 1987, Inositol trisphosphate and diacylglycerol: Two interacting second messengers. Ann. Rev. Biochem. 56:159-193

Beyer, E., D.L. Paul and D.A. Goodenough, 1990, Connexin family of gap junction proteins. J. Memb. Biol. 116:187-194

Bhardwaj, R., C.P. Page, G.R. May, and P.K. Moore, 1988, Endothelium-derived relaxing factor inhibits platelet aggregation in human whole blood in vitro and in the rat in vivo. Eur. J. Pharmacol. 157:83-91

Bhullar, R.P. and R.J. Haslam, 1988, G_n-proteins are distinct from *ras* p21 and other known low molecular mass GTP-binding proteins in the platelet. FEBS Lett. 237:168-172

Birnbaumer, L., 1990, G proteins in signal transduction. Ann. Rev. Pharmacol. Toxicol. 30:675-705

Bkaily, G., M. Peyrow, T. Yomamoto, A. Sculptoreanu, D. Jacques and N. Sperelakis, 1988, Macroscopic Ca^{2+}-Na^{+} and K^{+} currents in single heart and aortic cells. Moll. Cell. Biochem. 80: 59-72

Blair, I.A. and J. MacDermot, 1981, The binding of [^{3}H]prostacyclin to membranes of a neuronal somatic hybrid. Br. J. Pharmacol. 72:435

Böhme, E., Graf, H. and G. Schultz, 1978, Effects of sodium nitroprusside and other smooth muscle relaxants on cyclic GMP formation in smooth muscle and platelets. Adv. Cyclic Nucleotide Res. 9:131-143

Bowen, R. and R.J. Haslam, 1991, Effects of nitrovasodilators on platelet cyclic nucleotide levels in rabbit blood; Role for cyclic AMP in the synergistic inhibition of platelet function by SIN-1 and prostaglandin E_1. J. Cardiovasc. Pharmacol. *in press*

Braquet, P., L. Touqui, T.Y. Shen and B.B. Vargaftig, 1987, Perspectives in platelet-activating factor research. Pharmacol. Rev. 39:97-145

Brass, L.F., 1989, Regulation of biological responses in platelets in "Blood cells and Arteries in Hypertension and Atherosclerosis, Eds. Meyer, P. and Marche, P. pp.59-92, Raven Press, New York

Brass, L.F. and S.K. Joseph, 1985, A role for inositol triphosphate in intracellular Ca^{2+} mobilization and granule secretion in platelets. J. Biol. Chem. 260:15172-15179

Bredt, D.S. and S.H. Snyder, 1990, Isolation of nitric oxide synthase, a calmodulin-requiring enzyme. Proc. Natl. Acad. Sci. U.S.A. 87:682-685

Busse, R., A. Luckhoff, and E. Bassenge, 1987, Endothelium-derived relaxant factor inhibits platelet activation. Naunyn-Schmiedeberg's Arch. Pharmacol. 336:566-571

Byrne, N.G. and W.A. Large, 1987, The action of noradrenaline on single smooth muscle cells freshly dispersed from the guinea pig pulmonary artery. Br. J. Pharmacol. 91:89-94

Carlson, K.E., L.F. Brass and D.R. Manning, 1989, Thrombin and phorbol esters cause the selective phosphorylation of a G-protein other than G_i in human platelets. J. Biol. Chem. 264:13298-13305

Casey, P.J., H.K.W. Fong, M.I. Simon and A.G. Gilman, G_z, a guanine nucleotide-binding protein with unique biochemical properties. J. Biol. Chem. 265:2383-2390

Casnellie, J.E., H.E. Ives, J.D. Jamieson and P. Greengard, 1980, Cyclic GMP-dependent protein phosphorylation in intact medial tissue and isolated cells from vascular smooth muscle. J. Biol. Chem. 255:3770-3776

Cazenave, J.P., J. Benveniste and J.F. Mustard, 1979, Aggregation of rabbit platelets by platelet-activating factor is independent of the release reaction and of the arachidonate pathway and inhibited by membrane-active drugs. Lab. Invest. 41:275-285

Chadwick, C.C., A. Saito and S. Fleischer, 1990, Isolation and characterization of the inositol trisphosphate receptor from smooth muscle. Proc. Natl. Acad. Sci. U.S.A. 87:2132-2136

Charbonneau, H., 1990, Structure-function relationships among cyclic nucleotide phosphodiesterase, in "Cyclic Nucleotide Phosphodiesterases: Stucture, Regulation and Drug Action", Eds Beavo, J. and M.D. Houslay pp. 267-296, Wiley, Chichester

Charbonneau, H., N. Beier, K.A. Walsh and J.A. Beavo, 1986, Identification of a conserved domain among cyclic nucleotide phosphodiesterases from diverse species. Proc. Natl. Acad. Sci. U.S.A. 83:9308-9312

Charbonneau, H., R.B. Prusti, H.L. Trong, W.K. Sonnenburg, P.J. Mullaney, K.A. Walsh and J.A. Beavo, 1990, Identification of a noncatalytic cGMP-binding domain conserved in both the cGMP-stimulated and photoreceptor cyclic nucleotide phosphodiesterases. Proc. Natl. Acad. Sci, U.S.A. 87:288-292

Chen, C.-N., S. Denome and R.L. Davis, 1986, Molecular analysis of cDNA clones and the corresponding genomic coding sequences of the Drosophila dunce^{+} gene, the structural gene for cAMP phosphodiesterase. Proc. Natl. Acad. Sci. U.S.A. 83:9313-9317

Chignard, M., J.-P. LeCouedic, B.B. Vargaftig and J. Benveniste, 1980, Platelet-activating factor (PAF-Acether) secretion from platelets: Effect of aggregating agents. Br. J. Haematol. 46:455-464

Chinkers, M. and D.L. Garbers, 1989, The protein kinase domain of the ANP receptor is required for signaling. Science 245:1392-1394

Chinkers, M., D.L. Garbers, M.-S. Chang, D.G. Lowe, H. Chin, D.V. Goeddel and S. Schulz, 1989, A membrane form of guanylate cyclase is an atrial natriuretic peptide receptor. Nature 338:78-83

Chrisman, T.D., D.L. Garbers, M.A. Parks and J.G. Hardman, 1975, Characterization of particulate and soluble guanylate cyclases from rat lung. J. Biol. Chem. 250:374-381

Chuprun, J.K. and R.M. Rapoport, 1987, Nitroglycerin-induced desensitization of vascular smooth muscle may be mediated through cyclic GMP-disinhibition of phosphatidylinositol hydrolysis. Experientia 43:316-318

Clemetson, K.J. and E.F. Lüscher, Membrane glycoprotein abnormalities in pathological platelets. Biochim. Biophys. Acta 947:53-73

Clapp, L.H., M.B. Vivaudou, J.J. Singer and J.V. Walsh, 1987, A diacylglycerol analogue mimics the action of acetylcholine and substance P on calcium currents in freshly dissociated smooth muscle cells. J. Gen. Physiol. 90:13a

Collier, J. and P. Vallance, 1989, Endothelium-derived relaxing factor is an endogenous vasodilator in man. Br. J. Pharmacol. 97:639-641

Collins, P., T.M. Griffith, A. H. Henderson and M.J. Lewis, 1986, Endothelium-derived relaxing factor alters calcium fluxes in rabbit aorta: A cyclic guanosine monophosphate-mediated effect. J. Physiol. 381:427-437

Collins, S., M.A. Bolanowski, M.G. Caron, and R.J. Lefkowitz, 1989, Genetic regulation of β-adrenergic receptors. Ann. Rev. Physiol. 51:203-215

Conti, M.A. and R.S. Adelstein, 1987, The relationship between calmodulin binding and phosphorylation of smooth muscle myosin kinase by the catalytic subunit of 3':5'cAMP-dependent protein kinase. J. Biol. Chem. 256:3178-3181

Conti, M. and J.V. Swinnen, 1990, Structure and function of the rolipram-sensitive, low K_m cyclic AMP phosphodiesterase: A family of highly related enzymes, in "Cyclic Nucleotide Phosphodiesterases: Structure, Regulation and Drug Action" Eds Beavo, J.A. and M.D. Houslay pp. 243-266, Wiley, Chichester

Cornwell, T.L. and T.M. Lincoln, 1989, Regulation of intracellular Ca^{2+} levels in cultured vascular smooth muscle cells. Reduction of Ca^{2+} by atriopeptin and 8-bromo-cyclic GMP is mediated by cyclic GMP-dependent protein kinase. J. Biol. Chem. 264;1146-1155

Corsini, A., G.C. Folco, R. Fumagalli, S. Nicosia, M.A. Noe' and D Oliva, 1987, (5Z)-carbacyclin discriminates between prostacyclin-receptors coupled to adenylate cyclase in vascular smooth muscle and platelets. Br. J. Pharmacol. 90:255-261

Cox, A.C., R.C. Carroll, J.G. White and G.H.R. Rao, 1984, Recycling of platelet phosphorylation and cytoskeletal assembly. J. Cell. Biol. 98:8-15

Curtis, B.M. and W.A. Catterall, 1985, Phosphorylation of the calcium antagonist receptor of the voltage-sensitive calcium channel by cAMP-dependent protein kinase. Proc. Natl. Acad. Sci. U.S.A. 82:2528-2532

Dabrowska, R., D. Aromatorio, J.M.F. Sherry and D.J. Hartshorne, 1977, Composition of the myosin light chain kinase from chicken gizzard. Biochem. Biophys. Res. Commun. 78:1263-1272

Daniel, J.L., I.R. Molish, A.M. Rigmaiden and G. Stewart, 1984, Evidence for a role of myosin phosphorylation in the initiation of the platelet shape change response. J. Biol. Chem. 259:9826-9831

Davies, T., M.M.L. Davidson, M.D. McClenaghan, A. Say, and R.J. Haslam, 1976, Factors affecting platelet cyclic GMP levels during aggregation induced by collagen and by arachidonic acid. Thromb. Res. 9:387-405

Deanna, R., M. Ruzzene, M.G. Doni, F. Zoccarato and A. Alexandre, 1989, Cyclic GMP and nitroprusside inhibit the activation of human platelets by fluoroaluminate. Biochem. Biophys. Acta 1014:203-206

De Caterina, R., D. Giannessi, F. Crea, S. Chierchia, W. Bernini, P. Gazzetti, and A. L'Abbate, 1984, Inhibition of platelet function by injectable isosorbide dinitrate. Am. J. Cardiol. 53:1683-1687

De Caterina, R., D. Giannessi, W. Bernini and A. Mazzone, 1988, Organic nitrates: Direct antiplatelet effects and synergism with prostacyclin. Antiplatelet effects of organic nitrates. Thromb. Haemostas. 59:207-211

DeFeo, T.T. and K.G. Morgan, 1989, Calcium-force coupling mechanisms during vasodilator-induced relaxation of ferret aorta. J. Physiol. 412:123-133

Degerman, E., V.C. Manganiello, A.H. Newman, K.C. Rice and P Belfrage, 1988, Purification, properties and polyclonal antibodies for the particulate, low K_m cAMP phosphodiesterase from bovine adipose tissue. Sec. Mess. Phosphoproteins 12:171-182

DeJonge, H.R., 1981, Cyclic GMP-dependent protein kinase in intestinal brushborder. Adv. Cyclic Nucleotide Res. 14:315-333

Demopoulos, C.A., R.N. Pinckard and D.J. Hanahan, 1979, Platelet-activating factor. Evidence for 1-0-alkyl-2-acetyl-sn-glyceryl 3-phosphorylcholine as the active component (a new class of lipid chemical mediators). J. Biol. Chem. 254:9355-9358

De Nucci, G., R.J. Gryglewski, T.D. Warner and J.R. Vane, 1988, Receptor-mediated release of endothelium-derived relaxing factor and protacyclin from bovine aortic endothelial cells is coupled. Proc. Natl. Acad. Sci. U.S.A. 85:2334-2338

Deterre, P., J. Bigay, F. Forquet, M. Robert and M. Chabre, 1988, cGMP phosphodiesterase of retinal rods is regulated by two inhibitory subunits. Proc. Natl. Acad. Sci. U.S.A. 85:2424-2428

Diamond, J. and K.S. Blizzard, 1976, Effects of stimulant and relaxant drugs on tension and cyclic nucleotide levels in canine femoral artery. Mol. Pharmacol. 12:688-692

Dillon, P.F., M.O. Aksoy, S.P. Driska and R.A. Murphy, 1981, Myosin phosphorylation and the cross-bridge cycle in arterial smooth muscle. Science 211:495-497

DiSalvo, J., D. Gifford, C. Bialojan and J.C. Rüegg, 1983, An aortic spontaneously active phosphatase dephosphorylates myosin and inhibits actin-myosin interaction. Biochem. Biophys. Res. Commun. 111:906-911

Donnelly, T.E., 1978, Reconsideration of the multiple nucleotide phosphodiesterases in bovine heart. Biochem. Biophys. Res. Commun. 82:964-970

Draznin, M.B., R.M. Rapoport and F. Murad, 1986, Myosin light chain phosphorylation in contraction and relaxation of intact rat thoracic aorta. Int. J. Biochem. 18:917-928

Dutta-Roy, A.K. and A.K. Sinha, 1987, Purification and properties of prostaglandin E_1/prostacyclin receptor of human blood platelets. J. Biol. Chem. 262:12685-12691

Edelman, A.M., D.K. Blumenthal and E.G. Krebs, 1987, Protein serine/threonine kinases. Ann. Rev. Biochem. 56:567-613

Eglen, R.M. and R.L. Whiting, 1988, The action of prostanoid receptor agonists and antagonists on smooth muscle and platelets. Br. J. Pharmacol. 94:591-601

Ehrlich, B.E. and J. Watras, 1988, Inositol 1,4,5-trisphosphate activates a channel from smooth muscle sarcoplasmic reticulum. Nature 336:583-586

Enouf, J., F. Giraud, R. Brédoux, N. Bourdeau and S. Levy-Toledano, 1987, Possible role of a cAMP-dependent phosphorylation in the calcium release mediated by inositol 1,4,5-trisphosphate in human platelet membrane vesicles. Biochim. Biophys. Acta 928:76-82

Erneux, C., F. Miot, P.J.M. Van Haastert and B. Jastorff, 1985, The binding of cyclic nucleotide analogs to a purified cyclic GMP-stimulated phosphodiesterase from bovine adrenal tissue. J. Cyclic Nucleotide Protein Phosphorylation Res. 10:463-470

Feder, D. M.-J. Im, H.W. Klein, M. Hekman, A. Holzhofer, C. Dees, A. Levitzki, E.J.M. Helmreich and T. Pfeuffer, 1986, Reconstitution of β-adrenoreceptor-dependent adenylate cyclase from purified components. EMBO J. 5:1509-1514

Feinberg, H., W.C.Sandler, M. Scorer, G.C. Le Breton, B. Grossman and G.V.R. Born, 1977, Movement of sodium into human platelets induced by ADP. Biochim. Biophys. Acta 470:317-324

Feinstein, M.B., J.J. Egan, R.I. Sha'afi and J. White, 1983, The cytoplasmic concentration of free calcium in platelets is controlled by stimulators of cyclic AMP production (PGD_2, PGE_1, forskolin). Biochem. Biophys. Res. Commun. 113:598-604

Felbel, J., B. Trockur, T. Ecker, W. Landgraf and F. Hofmann, 1988, Regulation of cytosolic calcium by cAMP and cGMP in freshly isolated smooth muscle cells from bovine trachea. J. Biol. Chem. 263:16764-16771

Fenton, R.A., S.P. Bruttig, R. Rubio and R.M. Berne, 1982, Effects of adenosine on calcium uptake by intact and cultured vascular smooth muscle. Am. J. Physiol. 242:H797-H804

Fielder, V. B., 1982, Reduction of occlusive coronary artery thrombosis and myocardial ischemia by molsidomine in anesthetized dogs. Can. J. Physiol. Pharmacol. 60:104-1111

Fischmeister, R. and H.C. Hartzell, 1990, Regulation of calcium current by low K_m cyclic AMP phosphodiesterase in cardiac cells. Mol. Pharmacol. 38:426-433

Fox, J.E.B., 1987, The platelet cytoskeleton, in "Thrombosis and Haemostasis" Eds. Verstraete, M., J. Vermylen, R. Lijnen and J. Arnout pp.175-225, Louven University Press, Leuven

Fox, J.E.B. and M.C. Berndt, 1989, Cyclic AMP-dependent phosphorylation of glycoprotein Ibβ inhibits collagen-induced polymerization of actin in platelets. J. Biol. Chem. 264:9520-9526

Fox, J.E.B., A.K. Say and R.J. Haslam, 1979, Subcellular distribution of the different platelet proteins phosphorylated on exposure of intact platelets to ionophore A23187 or to prostaglandin E_1. Possible role of a membrane phosphopolypeptide in the regulation of calcium-ion transport. Biochem. J. 184:651-661

Fox, J.E.B., C.C. Reynolds and M.M. Johnson, 1987, Identification of glycoportein Ibβ as one of the major proteins phosphorylated during exposure of intact platelets to agents that activate cAMP-dependent protein kinase. J. Biol. Chem. 262:12627-12631

Flockerzi, V., H. Oeken, F. Hofmann, 1986, Purified dihydropyridine-binding site from skeletal muscle t-tubules is a functional calcium channel. Nature 323:66-68

Francis, S.H., M.K. Thomas and J.D. Corbin, 1990, Cyclic GMP-binding cyclic GMP-specific phosphodiesterase from lung, in "Cyclic Nucleotide Phosphodiesterases: Structure, Regulation and Drug Action" Eds. Beavo, J. and M.D. Houslay, pp. 117-140, Wiley, Chichester

Frearson, N., B.W.W. Focant and S.V. Perry, 1976, Phosphorylation of a light chain component of myosin from smooth muscle. FEBS Lett. 63:27-32

Freissmuth, M., P.J. Casey and A.G. Gilman, 1989, G-proteins control diverse pathways of transmembrane signaling. FASEB J. 3:2125-2131

Fujii, K., T. Ishimatsu and H. Kuriyama, 1986, Mechanism of vasodilation induced by α-human atrial natriuretic polypeptide in rabbit and guinea-pig renal arteries. J. Physiol. 377:315-332

Fuller, F., J.G. Porter, A.E. Arfsten, J. Miller, J.W. Schilling, R.M. Scarborough, J.A. Lewicki and D.B. Schenk, 1988, Arial natriuretic peptide clearance receptor: Complete sequence and functional expression of cDNA clones. J. Biol. Chem. 263:9395-9401

Furchgott, R.F. and J.V. Zawadzki, 1980, The obligatory role of endothelial cells in the relaxation of arterial smooth muscle by acetylcholine. Nature 288:373-376

Furlong, B., A. H. Henderson, M. J. Lewis and J. A. Smith, 1987, Endothelium-derived relaxing factor inhibits *in vitro* platelet aggregation. Br. J. Pharmacol. 90:687-692

Furukawa, K. and H, Nakamura, 1987, Cyclic GMP regulation of the plasma membrane (Ca^{2+}-Mg^{2+})ATPase in vascular smooth muscle. J. Biochem. 101:287-290

Furuya, M., M. Takehisa, Y. Minamitake, Y. Kitajima, Y. Hayashi, N. Ohnuma, T. Ishihara, N. Minamino, K. Kangawa and H. Matsuo, 1990, Novel natriuretic peptide, CNP, potently stimulates cyclic GMP production in rat cultured vascular smooth muscle cells. Biochem. Biophys. Res. Commun. 170:201-208

Gabella, G., 1984, Smooth muscle cell membrane and allied structures in: "Smooth Muscle Contraction", Ed. N.L. Stephens; Marcel Dekker, New York

Gallant, S., F.C. Kauffman and A.C. Brownie, 1974, Cyclic nucleotide phosphodiesterase activity in rat adrenal gland zones. Life Sci. 14:937-944

Garbers, D.L., 1979, Purification of soluble guanylate cyclase from rat lung. J. Biol. Chem. 254:240-243

Garbers, D.L. and E.W. Radany, 1981, Characteristics of the soluble and particulate forms of guanylate cyclase. Adv. Cyclic Nucleotide Res. 14:241-254

Garbers, D.L., H.D. Watkins, J.R. Hansbrough, A. Smith and K. S. Misono, 1982, The amino acid sequence and chemical synthesis of speract and of speract analogues. J. Biol. Chem. 257:2734-2737

Gardiner, S.M., A.M. Compton. T. Bennett, R.M.J. Palmer and S. Moncada, 1990, Control of regional blood flow by endothelium-derived nitric oxide. Hypertension 15:486-492

Garthwaite, J., S.L. Charles and R. Chess-Williams, 1988, Endothelium-derived relaxing factor release on activation of NMDA receptors suggests role as intercellar messenger in the brain. Nature 336:385-388

Gauthier, G.F., 1988, The Muscular Tissues in Cell and Tissue Biology, 6th Edition, L. Weiss; Urban and Schwarzenberg Inc., Baltimore

George, J.N., T.A. Reiman, J.L. Moake, R.K. Morgan, P.L. Cimo and D.A. Sears, 1981, Bernard-Soulier disease: a study of four patients and their parents. Br. J. Haematol. 48:459-467

Gerthoffer, W.T., M.A. Trevethick and R.A. Murphy, 1984, Myosin phosphorylation and cyclic adenosine 3',5'-monophosphate in relaxation of arterial smooth muscle by vasodilators. Circulation Res. 54:83-89

Gerzer, R., E. Böhme, F. Hofmann and C. Schultz, 1981, Soluble guanylate cyclase purified from bovine lung contains heme and copper. FEBS Lett. 132:71-74

Gerzer, R., B. Karrenbrock, W. Siess, and J.-M. Heim, 1988, Direct comparison of the effects of nitroprusside, SIN-1, and various nitrates on platelet aggregation and soluble guanylate cyclase activity. Thromb. Res. 52:11-21

Gettys, T.W., P.F. Blackmore, J.B. Redmon, S.J. Beebe and J.D. Corbin, 1987, Short-term feedback regulation of cAMP by accelerated degradation in rat tissues. J. Biol. Chem. 262:333-339

Gibson, A., S. Mirzazadeh, A.J. Hobbs and P.K. Moore, 1990, L-N^G-monomethyl arginine and L-N^G-nitro arginine inhibit non-adrenergic, non-cholinergic relaxation of the mouse anococcygeus muscle. Br. J. Pharmacol. 99:602-606

Gillespie, E., 1988, Anagrelide: a potent and selective inhibitor of platelet cyclic AMP phosphodiesterase enzyme activity. Biochem. Pharmacol. 37:2866-2868.

Gillespie, P.G., 1990, Phosphodiesterase in visual transduction by rods and cones in "Cyclic Nucleotide Phosphodiesterases: Structure, Regulation and Drug Action" Eds. Beavo, J. and M.D. Houslay pp. 163-184, Wiley, Chichester

Gillespie, J.S., X. Liu and W. Martin, 1989, The effects of L-arginine and N^G-monomethyl L-arginine on the response of the rat anococcygeus muscle to NANC nerve stimulation. Br. J. Pharmacol. 98:1080-1082

Glusa, E., F. Markwardt and J. Stürzebecher, 1974, Effects of sodium nitroprusside and other pentacyanonitrosyl complexes on platelet aggregation. Haemostasis 3:249-256

Goldman, W.F., W.G. Wifr and M.P. Blaustein, 1989, Effects of activation on distribution of Ca^{2+} in single arterial smooth muscle cells. Circ. Res. 64:1019-1029

Grant, P.G. and R.W. Colman, 1984, Purification and characterization of a human platelet cyclic nucleotide phosphodiesterase. Biochemistry 23:1801-1807

Grant, P.G., A.F. Mannarino and R.W. Colman, 1988, cAMP-mediated phosphorylation of the low K_m cAMP phosphodiesterase markedly stimulates its catalytic activity. Proc. Natl. Acad. Sci. U.S.A. 85:9071-9079

Grant, P.G., A.F. Mannarino and R.W. Colman, 1990, Purification and characterization of a cyclic GMP-stimulated cyclic nucleotide phosphodiesterase from the cytosol of human platelets. Thrombosis Res. 59:105-119

Gresele, P., C. Zola, H. Deckymyn, J. Arnout, J. Vermylen and M. Verstraete, 1983, Dipyridamole inhibits platelet aggregation in whole blood. Thromb. Haemostas. 50:852-856

Griendling, K.K., S.E. Rittenhouse, T.A. Brock, L.S. Ekstein, M.A. Gimbrone and R.W. Alexander, 1986, Sustained diacylglycerol formation from inositol phospholipids in angiotension II-stimulated vascular smooth muscle cells. J. Biol. Chem. 261:5901-5906

Hack, N., M. Croset and N Crawford, 1986, Studies on the bivalent-cation-activated ATPase activities of highly purified human platelet surface and intracellular membranes. Biochem. J. 233:661-668

Hai, C-.M. and R.A. Murphy, 1988, Cross-bridge phosphorylation and regulation of latch state in smooth muscle. Am. J. Physiol. 254:C99-C106

Hai, C.-M. and R.A. Murphy, 1989, Ca^{2+}, crossbridge phosphorylation and contraction. Ann Rev. Physiol. 51:285-298

Halbrügge, M. and U. Walter, 1989, Purification of a vasodilator-regulated phosphoprotein from human platelets. Eur. J. Biochem. 185:41-50

Halbrügge, M., C. Friedrich, M. Eigenthaler, P. Schanzenbacher and U. Walter, 1990, Stoichiometric and reversible phosphorylation of a 46 kDa protein in human platelets in response to cGMP- and cGMP-elevating vasodilators. J. Biol. Chem. 265:3088-3093

Hall, A., 1990, The cellular functions of small GTP-binding proteins. Science 249:635-640 Halushka, P.V., D.E. Mais, P.R. Mayeux and T.A. Morinelli, 1989, Thromboxane, prostaglandin and leukotriene receptors. Ann. Rev. Pharmacol. Toxicol. 10:213-239

Hamet, P. and J.-F. Coquil, 1978, Cyclic GMP binding and cyclic GMP phosphodiesterase in rat platelets. J. Cyclic Nucleotide Res. 4:281-290

Hamet, P., S.C. Pang and J. Tremblay, 1989, Atrial natriuretic factor-induced egression of cyclic guanosine 3':5'-monophosphate in cultured vascular smooth muscle and endothelial cells. J. Biol. Chem. 264:12364-12369

Hamet, P. and J. Tremblay, 1988, Platelet cGMP-binding phosphodiesterase. Methods Enzymol. 159:710-722

Hamet, P., J. Tremblay, S.C. Pang, R. Garcia, G. Thibault, J. Cantin, G. Gutkowska and J. Genest, 1984, Effect of native and synethetic atrial natriuretic factor on cyclic GMP. Biochem. Biophys. Res. Commun. 123:515-527

Hansbrough, J.R. and D.L. Garbers, 1981, Speract: Purification and characterization of a peptide associated with eggs that activates spermatozoa. J. Biol. Chem. 256:1447-1452

Hardman, J.G., 1984, Cyclic nucleotides and regulation of vascular smooth muscle. J. Cardiovasc. Pharmacol. 6:S639-S645

Harmon, J.T. and G.A. Jamieson, 1986, Activation of platelets by α-thrombin is a receptor-mediated event. D-phenylyalanyl-L-propyl-L-arginine chloromethyl ketone-thrombin, but not *N*-tosyl-L-lysine chloromethyl ketone-thrombin, binds to the high affinity thrombin receptor. J. Biol. Chem. 261:15928-15933

Harper, J.F., and G. Brooker, 1975, Femtomole sensitive radioimmunoassay for cyclic AMP and cyclic GMP after 2'O acetylation by acetic anhydride in aqueous solution. J. Cyclic Nucleotide Res. 1:207-218

Harris, A.L., A.M. Grant, P.J. Silver, D.B. Evans and A.A. Alousi, 1989, Differential vasorelaxant effects of milrinone and amrinone on contractile responses of canine coronary cerebral and renal arteries. J. Cardiovasc. Pharmacol. 13:238-244

Harrison, S.A., M.L. Chang and J.A. Beavo, 1986a, Differential inhibition of cardiac cyclic nucleotide phosphodiesterase isozymes by cardiotonic drugs. Circulation 73 (S3):109-116

Harrison, S.A., D.H. Reifsnyder, B. Gallis, G.G. Cadd and J.A. Beavo, 1986b, Isolation and characterization of bovine cardiac muscle cGMP-inhibited phosphodiesterase: A receptor for new cardiotonic drugs. Mol. Pharmacol. 29:506-514

Harteneck, C., D. Koesling, A. Söling, G. Schultz and E. Böhme, 1990, Expression of soluble guanylyl cyclase: catalytic activity requires two enzyme subunits. FEBS Lett. 272:221-223

Hartzell, H.C. and R. Fischmeister, 1986, Opposite effects of cyclic GMP and cyclic AMP on Ca^{2+} currents in single heart cells. Nature 323:273-275

Hashimoto, K., N. Taira, M. Hirata and M. Kokubun, 1971, The mode of hypotensive action of newly synthesized sydnonimine derivatives. Arzneim. Forsch. 21:1329-1332

Hashimoto, T., M. Hirata, T. Itoh, Y. Kanamura and H. Kuriyama, 1986, Inositol 1,4,5-trisphosphate activates pharmacomechanical coupling in smooth muscle of the rabbit mesenteric artery. J. Physiol. 370:605-618

Haslam, R.J., 1987, Signal transduction in platelet activation in "Thrombosis and Haemostasis" Eds.Verstraete, M., J. Vermylen, R. Lijnen, J. Arnout pp. 147-174, Leuven University Press, Leuven

Haslam, R.J., and M.M.L. Davidson, 1982, Roles of cyclic AMP and cyclic GMP in the inhibition of platelet function by nitroprusside and phosphodiesterase inhibitors. Circulation 66:II-291

Haslam, R.J., and M.D. McClenaghan, 1981, Measurement of circulating prostacyclin. Nature 292:364-366

Haslam, R.J., and G.M. Rosson, 1975, Effects of adenosine on levels of adenosine cyclic 3',5'-monophosphate in human blood platelets in relation to adenosine incorporation and platelet aggregation. Mol. Pharmacol. 11:528-544

Haslam, R.J., M.M.L. Davidson, M. Culty and J.K. Hrbolich, 1988, Role of a guanine-nucleotide binding protein in the agonist-stimulated hydrolysis of platelet phosphatidylinositides by phopholipase C in "Biochemistry and Physiopathology of Platelet Membranes" Eds. Margurie, G, and R.F.A. Zwaal, pp. 11-22, J. Libbey Eurotext, Montrouge

Haslam, R.J., M.M.L. Davidson, T. Davies, J.A. Lynham and M.D. McClenaghan, 1978, Regulation of blood platelet function by cyclic nucleotides. Adv. Cyclic Nucleotide Res. 9:533-552

Haslam, R.J., M.M.L. Davidson, and J.V. Desjardins, 1976, Inhibition of adenylate cyclase by adenosine analogues in preparations of broken and intact human platelets. Evidence for the unidirectional control of platelet function by cyclic AMP. Biochem. J. 176:83-95

Haslam, R.J., S.E. Salama, J.E.B. Fox, J.A. Lynham, and M.M.L. Davidson, 1980, Roles of cyclic nucleotides and of protein phosphorylation in the regulation of platelet function, in Platelets: Cellular Response Mechanisms and their Biological Significance Eds. Rotman A., F.A. Meyer, C. Gitler and A. Silberberg, pp. 213-231, Wiley, Chichester

Hassid, A., 1986, Atriopeptin II decreases cytosolic free Ca in cultured vascular smooth muscle cells. Am. J. Physiol. 251:C681-C686

Hathaway, D.R., M.V. Konicki and S.A. Coolican, 1985, Phosphorylation of myosin light chain kinase from vascular smooth muscle by cAMP and cGMP-dependent protein kinases. J. Mol. and Cell. Cardiol. 17:841-850

Hathaway, D.R., C.R. Eaton and R.S. Adelstein, 1987, Regulation of human platelet myosin light chain kinase by the catalytic subunit of cyclic AMP-dependent protein kinase. Nature 291:252-254

Hawkins, D.R., B.O. Meyrick, and J.J. Murray, 1988, Activation of guanylate cyclase and inhibition of platelet aggregation by endothelium-derived relaxing factor released from cultured cells. Biochim. Biophys. Acta 969:289-296

Headrick, J.P. and R.M. Berne, 1990, Endothelium-dependent and -independent relaxations to adenosine in guinea pig aorta. Am. J. Physiol. 259:H62-H67

Herlihy, J.T., E.L. Bockman, R.M. Berne and R. Rubio, 1976, Adenosine relaxation of isolated vascular smooth muscle. Am. J. Physiol. 230:1239-1243

Heyworth, C.M., A.V. Wallace and M.D. Houslay. 1983, Insulin and glucagon regulate the activation of two distinct membrane-bound cAMP phosphodiesterases in hepatocytes. Biochem. J. 214:99-110

Hettasch, J.M. and G.C. Le Breton, 1987, Modulation of Ca^{2+} fluxes in isolated platelet vesicles: Effects of cAMP-dependent protein kinase and protein kinase inhibitors on Ca^{2+} sequestration and release. Biochim. Biophys. Acta 931:49-58

Hibbs, J.B., R.R. Taintor and Z. Vavrin, 1987a, Macrophage cytotoxicity: role for L-arginine deiminase and imino nitrogen oxidation to nitrite. Science 235:473-480

Hibbs, J.B., Z. Vavrin and R.R. Taintor, 1987b, L-arginine is required for expression of the activated macrophage effector mechanism causing selective metabolic inhibition in target cells. J. Immunol. 138:550-558

Hidaka, H. and T. Asano, 1976, Human blood platelet 3':5'-cyclic nucleotide phosphodiesterase. Isolation of low-K_m and high K_m phosphodiesterase. Biochim. Biophys. Acta 429:485-497

Hidaka, H. and T. Endo, 1984, Selective inhibitors of three forms of cyclic nucleotide phosphodiesterase: Basis and potential clinical implications. Adv. Cyclic Nucleotide Protein Phosphorylation Res. 16:245-259

Hidaka, H., H. Hayashi, H. Kohri, Y. Kimura, T. Hosokawa, T. Igawa and Y. Saitoh, 1979, Selective inhibitor of platelet cyclic adenosine monophosphate phosphodiesterase, cilostamide, inhibits platelet aggregation. J. Pharmacol. Exp. Ther. 211:26-30

Hirata, M., K.P. Kohse, C.-H. Chang, T. Ikebe and F. Murad, 1990, Mechanism of cyclic GMP inhibition of inositol phosphate formation in rat aorta segments and cultured bovine aortic smooth muscle cells. J. Biol. Chem. 265:1268-1278

Hofmann, F. and V. Flockerzi, 1983, Characterization of phosphorylated and native cGMP-dependent protein kinase. Eur. J. Biochem. 130:599-603

Hofmann, F. and G. Sold, 1972, A protein kinase activity from rat cerebellum stimulated by guanosine 3':5'-monophosphate. Biochem. Biophys. Res. Commun. 49:1100-1107

Hofmann, F., H.P. Gensheimer and C. Gobel, 1985, cGMP-dependent protein kinase: Autophosphorylation changes the characteristics of binding site I. Eur. J. Biochem. 147:361-365

Hogan, J. C., M. J. Lewis, and A. H. Henderson, 1988, *In vivo* EDRF activity influences platelet funtion. Br. J. Pharmacol. 94:1020-1022

Hokin, L.E. and M.R. Hokin, 1955, Effects of acetylcholine on phosphate turnover in phospholipid of brain cortex in vitro. Biochim. Biophys. Acta 16:229-237

Hosey, M.M. and M. Lazdunski, 1988, Calcium Channels: Molecular pharmacology, structure and regulation. J. Membrane Biol. 104:81-105

Houslay, M.D. and E. Kilgour, 1990, Cylic nucleotide phosphodiesterases in liver: A review of their characterization, regulation by insulin and glucagon and their role in controlling intracellular cyclic AMP concentrations in "Cyclic Nucleotide Phosphodiesterases: Structure, Regulation and Drug Action, Eds. Beavo, J. and M.D. Houslay, pp. 105-224, Wiley, Chichester

Howrowski, R. and M. Sastre-Y-Hernandez, 1985, Clinical effects of the neurotropic selective phosphodiesterase inhibitor rolipram in depressed patients: Global evaluation of the preliminary reports. Curr. Ther. Res. 38:23-29

Hrbolich, J.K., M. Culty and R.J. Haslam, 1987, Activation of phospholipase C associated with isolated rabbit platelet membranes by guanosine 5'-[γ-thio]triphosphate and by thrombin in the presence of GTP. Biochem. J. 243:457-465

Huggins, J.P., E.A. Cook, J.R. Piggott, T.J. Mattincley and P.J. England, 1989, Phospholamban is a good substrate for cGMP-dependent protein kinase in vitro but not in intact cardiac or smooth muscle. Biochem. J. 260:829-835

Hwang, S.-B., C.-S.C. Lee, M.J. Cheah and T.Y. Shen, 1983, Specific receptor sites for 1-0-alkyl-2-0-acetyl-sn-glycero-3-phosphocholine (platelet activating factor) on rabbit platelet and guinea pig smooth muscle membranes. Biochemistry 22:4756-4763

Hwang, S.-B., M.-H. Lam and S.-S. Pong, 1986, Ionic and GTP regulation of binding of platelet-activating factor to receptors and platelet-activating factor-induced activation of GTPase in rabbit platelet membranes. J. Biol. Chem. 261:532-537

Hwong, K.S. and C. van Breeman, 1987, Effect of dB-cAMP and forskolin on the ^{45}Ca influx, net Ca uptake and tension in rabbit aortic smooth muscle. Eur. J. Pharmacol. 134:155-162

Ignarro, L. J., 1989a, Endothelium-derived nitric oxide: actions and properties. FASEB J. 3:31-36

Ignarro, L.J., 1989b, Heme-dependent activation of soluble guanylate cyclase by nitric oxide: Regulation of enzyme activity by porphyrins and metalloporphyrins. Sem. Hematol. 26:63-76

Ignarro, L.J., G.M. Buga, K.S. Wood, R.E. Byrns, and G. Chaudhuri, 1987, Endothelium-derived relaxing factor produced and released from artery and vein is nitric oxide. Proc. Natl. Acad. Sci. U.S.A. 84:9265-9269

Ignarro, L.J., P.A. Bush, G.M. Buga, K.S. Wood, J.M. Fukuto and J. Rajfer, 1990, Nitric oxide and cyclic GMP formation upon electrical field stimulation cause relaxation of corpus cavernosum smooth muscle. Biochem. Biophys. Res. Commun. 170: 843-850

Imai, A., H. Hattori, M. Takahashi and Y. Nozawa, 1983, Evidence that cyclic AMP may regulate Ca^{2+}-mobilization and phospholipases in thrombin-stimulated human platelets. Biochem. Biophys. Res. Commun. 112:693-700

Imaoka T., J.A. Lynham and R.J. Haslam, 1983, Purification and characterization of the 47,000-dalton protein phosphorylated during degranulation of human platelets. J. Biol. Chem. 258:11404-11414

Ishido, M., T. Fujita, M. Shimonaka, T. Saheki, S. Ohuchi, T. Kune, I. Ishigaki, and S. Hirose, 1989, Inhibition of atrial natriuretic peptide-induced cyclic GMP accumulation in the bovine endothelial cells with anti-atrial natriuretic peptide receptor antiserum. J. Biol. Chem. 264:641-645

Itoh, T., H. Kuriyama and H . Ueno, 1983, Mechanisms of the nitroglycerin-induced vasodilation in vascular smooth muscle of the rabbit and pig. J. Physiol. 343:233-252

Itoh, T., Y. Kanmura, H. Kuriyama and T. Sasaguri, 1985, Nitroglycerin- and isoprenaline-induced vasodilation: assessment from the actions of cyclic nucleotides. Br. J. Pharmacol. 84:393-406

Ives, H.E., J.E. Casnellie, P. Greengard and J.D. Jamieson, 1980, Subcellular localization of cyclic GMP-dependent protein kinase and its substrates in vascular smooth muscle. J. Biol. Chem. 255:3777-3785

Jakobs, K. H., E. Böhme, and G. Schultz, 1976, Determination of cyclic GMP in biological material, in Eukaryotic Cell Function and Growth Eds. Dumont, J.E., Brown B.L., and Marshall N.J., pp. 295-311, Plenum, New York

Johnson, R.M. and T.M. Lincoln, 1985, Effects of nitroprusside, glyceryl trinitrate, and 8-bromo cyclic GMP on phosphorylase a formation and myosin light chain phosphorylation in rat aorta. Mol. Pharmacol. 27:333-342

Kai, H., H. Kanaide, T. Matsumoto, Y. Shogakiuchi and M. Nakamura, 1987a, Adenosine decreases intracellular free calcium concentrations in cultured vascular smooth muscle cells from the rat. FEBS Lett. 212:119-122

Kai, H., H. Kanaide, T. Matsumoto and M. Nakamura, 1987b, 8-Bromo-guanosine 3':5'-cyclic monophosphate decreases intracellular free calcium concentration in cultured vascular smooth muscle cells from rat aorta. FEBS Lett. 221:284-288

Kamisaki, Y., S. Saheki, M. Nakane, J.A. Palmieri, T. Kuno, B.Y. Chang, S.A. Waldman and F. Murad, 1986, Soluble guanylate cyclase from rat lung exists as a heterodimer. J. Biol. Chem. 261:7236-7241

Kamm, K.E. and J.T. Stull, 1985, The function of myosin and myosin light chain kinase phosphorylation in smooth muscle. Ann. Rev. Pharmacol. Toxicol. 25:593-620

Karachi, H., 1989, Ca^{2+} localization and sensitivity in vascular smooth muscle. Trends Pharmacol. Sci. 10:320-325

Käser-Glanzmann, R., E. Gerber and E.F. Lüscher, 1979, Regulation of the intracellular calcium level in human blood platelets: Cyclic adenosine 3',5' monophosphate dependent phosphorylation of a 22,000-dalton component in isolated Ca^{2+}-accumulating vesicles. Biochim. Biophys. Acta 558:344-347

Katada, T. and M. Ui, 1982, Direct modification of the membrane adenylate cyclase system by islet-activating protein due to ADP-ribosylation of a membrane protein. Proc. Natl. Acad. Sci. U.S.A. 79:3129-3133

Katada, T., A.G. Gilman, Y. Watanabe, S. Bauer and K.H. Jacobs, 1985, Protein kinase C phosphorylates the inhibitory guanine-nucleotide-binding regulatory component and apparently suppresses its function in hormonal inhibition of adenylyl cyclase. Eur. J. Biochem. 151:431-437

Kaufman, R.F., V.G. Crowe, B.G. Utterback and D.W. Robertson, 1986, LY 195115: A potent, selective inhibitor of cyclic nucleotide phosphodiesterase located in the sarcoplasmic reticulum. Mol. Pharmacol. 30:609-619

Kawa, K., 1990a, Voltage-gated calcium and potassium currents in megakaryocytes dissociated from guinea-pig bone marrow. J. Physiol. 431:187-206

Kawa, K., 1990b, Guinea-pig megakaryocytes can respond to external ADP by activating Ca^{2+}-dependent potassium conductance. J. Physiol. 431:207-224

Kawahara, Y., J. Yamanishi and H. Fukuzaki, 1984, Inhibitory action of guanosine 3',5'-monophosphate on thrombin-induced calcium mobilization in human platelets. Thromb. Res. 33:203-209

Keilin, D., and E. F. Hartree, 1937, Reaction of nitric oxide with haemoglobin and methaemoglobin. Nature 139:548-551

Keith, R.A., A.M. Burkman, T.D. Sokoloski and R.H. Fertel, 1982, Vascular tolerance to nitroglycerin and cyclic GMP regeneration in rat aortic smooth muscle. J. Pharmacol. Exp. Ther. 221:525-531

Keravis, T.M., B.H. Duemler and J.M. Wells, 1987, Calmodulin sensitive phosphodiesterase of porcine cerebral cortex: Kinetic behaviour, calmodulin activation, and stability. J. Cyclic Nucleotide Protein Phosphorylation Res. 11:363-372

Kikkawa, U., A. Kishimoto and Y. Nishizuka, 1989, The protein kinase C family: heterogeneity and its implications. Ann. Rev. Biochem. 58:31-44

Kimura, H. and F. Murad, 1975a, Two forms of guanylate cyclase in mammalian tissues and possible mechamisms for their regulation. Metabolism 24:439-445

Kimura, H. and F. Murad, 1975b, Localization of particulate guanylate cyclase in plasma membranes and microsomes of rat liver. J. Biol. Chem. 250:4810-4817

Köesling, D., J. Herz, H. Gausepohl, F. Niroomand, K.-D. Hinsch, A. Mülsch, E. Böhme, G. Schultz and R. Frank, 1988, The primary structure of the 70 kDa subunit of bovine soluble guanylate cyclase. FEBS Lett. 239:29-34

Komori, S. and T.B. Bolton, 1988, Actions of guanine nucleosides and cyclic nucleotides on calcium stores in single patch-clamped smooth muscle cells from rabbit portal vein. Br. J. Pharmacol. 97:973-982

Kovacs, T., A. Tordai, I. Szasz, B. Sarkadi and G. Gardos, 1990, Membrane depolarization inhibits thrombin-induced calcium influx and aggregation in human platelets. FEBS Lett. 266:171-174

Kroll, M.H. and A.I. Schafer, 1989, Biochemical mechanisms of platelet activation. Blood 74:1181-1195

Krupinski, J., F. Coussen, H.A. Bakalyar, W.-J. Tang, P.G. Feinstein, K. Orth, C. Slaughter, R.R. Reed and A.G. Gilman, 1989, Adenylyl cyclase amino acid sequence: Possible channel- or transporter-like structure. Science 244:1558-1564

Kubota, M., J.M. Moseley, L. Butera, G.J. Dusting, P.S. MacDonald and T.J. Martin, 1985, Calcitonin gene-related peptide stimulates cyclic AMP formation in rat aortic smooth muscle cells. Biochem. Biophys. Res. Commun. 132:88-94

Kukovetz, W.R. and S. Holzmann, 1986, Cyclic GMP as the mediator of molsidomine-induced vasodilation. Eur. J. Pharmacol. 122:103-109

Kukovetz, W.R., S. Holzmann, A. Wurm and G. Pöch, 1979, Evidence for cyclic GMP-mediated relaxant effects of nitro-compounds in coronary smooth muscle. Naunyn-Schmiedeberg's Arch. Pharmacol. 310:129-138

Kuno, T., J.W. Andresen, Y. Komisaki, S.A. Waldman, L.Y. Chong, S. Sakeki, D.C. Leitman, M. Nakane and F. Murad, 1986, Co-purification of an atrial natriuretic factor receptor and particulate guanylate cyclase from rat lung. J. Biol. Chem. 261:5877-5823

Kuo, J.F. and P. Greengard, 1970, Cyclic nucleotide-dependent protein kinase. VI: Isolation and partial purification of a protein kinase activated by guanosine 3',5'-monophosphate. J. Biol. Chem. 245:2493-2498

Kurtz, T.W., M. Montano, L. Chan and P. Kabra, 1989, Molecular evidence of genetic heterogeneity in Wistar-Kyoto rats: Implications for research with the spontaneously hypertensive rat. Hypertension 13:188-192

Kwan, C.Y. and R.M.K.W. Lee, 1990, Changes of β-adrenoceptors in the aortic muscle cells of spontaneously hypertensive rats. Can. J. Physiol. Pharmacol. 68:1461-1468

Lang, D. and M.J. Lewis, 1989, Endothelium-derived relaxing factor inhibits the formation of inositol trisphosphate by rabbit aorta. J. Physiol. 411:45-52

Lapetina, E.G., 1990, The signal transduction induced by thrombin in human platelets. FEBS Lett. 268:400-404

Larsen, N.E. and E.R. Simons, 1987, Preparation and application of a photoreactive thrombin analogue: binding to human platelets. Biochemistry 20:4141-4147

Lee, M.A., R. E. West and J. Moss, 1988, Atrial natriuretic factor reduces cyclic adenosine monophosphate content of human fibroblasts by enhancing phosphodiesterase activity. J. Clin. Invest. 82:388-393

Leigh, P.J., W.A. Cramp and J. MacDermot, 1984, Identification of the prostacyclin receptor by radiation inactivation. J. Biol. Chem. 259:12431-12436

Le Peuch, C.J., D.A.M. Le Peuch, S. Katz, J.G. Demaille, M.T. Hincke, R. Bredoux, J. Enouf, S. Levy-Toledano and J. Caen, 1983, Regulation of calcium accumulation and effluxes from platelet vesicles: Possible role for cyclic-AMP-dependent phosphorylation and calmodulin. Biochim. Biophys. Acta 731:456-464

Lerea, K.M., J.A. Glomset and E.G. Krebs, 1987, Agents that elevate cAMP levels in platelets decrease thrombin binding. J. Biol. Chem. 262:282-288

Levin, R.I., B.B. Weksler and E.A. Jaffe, 1982, The interaction of sodium nitroprusside with human endothelial cells and platelets: nitroprusside and prostacyclin synergistically inhibit platelet function. Circulation 66:1299-1307

Levitzki, A., 1987, Regulation of hormone-sensitive adenylate cyclase. Trends Pharmacol. Sci. 8:299-303

Lidbury, P.S., E. Antunes, G. de Nucci and J.R. Vane, 1989, Interactions of iloprost and sodium nitroprusside on vascular smooth muscle and platelet aggregation. Br. J. Pharmacol. 98:1275-1280

Limbird, L.E., 1988, Receptors linked to inhibition of adenylate cyclase: additional signaling mechanisms. FASEB J. 2:2686-2695

Lincoln, T.M., 1983, Effects of nitroprusside and 8 Br-cyclic GMP on the contractile activity of the rat aorta. J. Pharmacol. Exp. Ther. 224:100-117

Lincoln, T.M., 1989, Cyclic GMP and mechanisms of vasodilation. Pharmac. Ther. 47:479-502

Lincoln, T.M., T.L. Cornwell and A.E. Taylor, 1990, cGMP-dependent protein kinase mediates the reduction of Ca^{2+} by cAMP in vascular smooth muscle cells. Am. J. Physiol. 258:C399-C407

Lindgren, S., A. Rascon, E. Degerman, P. Belfrage, V. Manganiello and K.-E. Andersson, 1990a, Identification of the cGMP-inhibited low K_m cAMP phosphodiesterase in rat aortic smooth muscle. Eur. J. Pharmacol. 183:806.

Lindgren, S.H.S., T.L.G. Andersson, E. Vinge and K.E. Andersson, 1990b, Effects of isozyme-selective phosphodiesterase inhibitors on rat aorta and human platelets: smooth muscle tone, platelet aggregation and cAMP levels. Acta Physiol. Scand. 140:209-219

Livi, G.P., P. Kmetz, M.M. McHale, L.B. Cieslinski, G.M. Sathe, D.P. Taylor, R.L. Davis, T.J. Torphy and J.M. Balcarek, 1990, Cloning and expression of cDNA for a human low K_m, rolipram-sensitive cyclic AMP phosphodiesterase. Mol. Cell. Biol. 10:2678-2686

Londos, C. and J. Wolff, 1977, Two distinct adenosine-sensitive sites on adenylate cyclase. Proc. Natl. Acad. Sci. U.S.A. 74:5482-5486

Lopez, J.A., D.W. Chung, K. Fujikawa, F.S. Hagen, E.W. Davies and G.J. Gilman, The α and β chains of human platelet glycoprotein Ib are both transmembrane proteins containing a leucine-rich amino acid sequence. Proc. Natl. Acad. Sci. U.S.A. 85: 2135-2139

Loscalzo, J., 1985, N-Acetylcysteine potentiates inhibition of platelet aggregation by nitroglycerin. J. Clin. Invest. 76:703-708

Lowe, D.G., M.-S. Chang, R. Hellmiss, E. Chen, S. Singh, D.L. Garbers and D.V. Goeddel, 1989, Human atrial natriuretic peptide receptor defines a new paradigm for second messenger signal transduction. EMBO J. 8:1377-1384

Lowry, O.H., N.J. Rosebrough, A.L. Farr and R.J. Randall, 1951, Protein measurement with the Folin phenol reagent. J. Biol. Chem. 193:265-275

Lugnier, C., A. Stierlé, A. Beretz, P. Schoeffter, A. Lebec, C.-G. Wermuth, J.-P. Cazenave and J.-C. Stoclet, 1983, Tissue and substrate specificity of inhibition by alkoxy-aryl-lactams of platelet and arterial smooth muscle cyclic nucleotide phosphodiesterases; relationship to pharmacological activity. Biochem. Biophys. Res. Commun. 113:954-959

Macdonald, P.S., M.A. Read and G.J. Dusting, 1988, Synergistic inhibition of platelet aggregation by endothelium-derived relaxing factor and prostacyclin. Thromb. Res. 49:437-448

MacFarland, R.T., B.D. Zelus and J.A. Beavo, 1991, High concentrations of a cGMP-stimulated phosphodiesterase mediate ANP-induced decreases in cAMP and steroidogenesis in adrenal glomerulosa cells. J. Biol. Chem. 266:136-142

Machleidt, C., P. Rose and U. Mittmann, 1985, Prevention of platelet aggregation with a phosphodiesterase inhibitor RX-RA 69. Thromb. Res. 37:595-604

MacPhee, C.H., S.A. Harrison and J.A. Beavo, 1986, Immunological identification of the major platelet low K_m cAMP phosphodiesterase: Probable target for anti-thrombotic agents. Proc. Natl. Acad. Sci. U.S.A. 83:6660-6668

MacPhee, C.H., D.H. Reifsnyder, T.A. Moore, K.M. Lerea and J.A. Beavo, 1988, Phosphorylation results in activation of a cAMP phosphodiesterase in human platelets. J. Biol. Chem. 263:10353-10358.

Magliola, L. and A.W. Jones, 1990, Sodium nitroprusside alters Ca^{2+} flux components and Ca^{2+}-dependent fluxes of K^+ and Cl^- in rat aorta. J. Physiol. 421:411-424

Majerus, P.W., D.M. Tollefsen and M.A. Shuman, 1976, The interaction of platelets with thrombin in "Platelets in Biology and Pathology" Ed. Gordon, J.L., North-Holland Publishing, Amsterdam

Majerus, P., T.S. Ross, T.W. Cunningham, K.K. Caldwell, A.B. Jefferson and V.S. Bansal, 1990, Recent insights in phosphatidylinositol signaling. Cell 63:459-465

Manganiello, V.C., T. Tanaka and S. Murashima, 1990, Cyclic GMP-stimulated cyclic nucleotide phosphodiesterases, in "Cyclic nucleotide Phosphodiesterases: Structure, Regulation and Drug Action" Eds. Beavo, J. and M.D. Houslay, pp. 61-85, Wiley, Chichester.

Marchmont, R.J., S.R. Ayad and M.D. Houslay, 1981, Purification and properties of the insulin stimulated cyclic AMP phosphodiesterase from rat liver plasma membranes. Biochem. J. 195:645-652

Marks, A.R., P. Tempst, C.C. Chadwick, L. Riviere, S. Fleischer and B. Nadal-Ginard, 1990, Smooth muscle and brain inositol 1,4,5-trisphosphate receptors are structurally and functionally similar. J. Biol. Chem. 265:20719-20722

Marletta, M.A., P.S. Yoon, R. Iyengar, C.D. Leaf and J.S. Wishnok, 1988, Macrophage oxidation of L-arginine to nitrite and nitrate: Nitric oxide is an intermediate. Biochemistry 27:8706-8711

Martin, W., R.F. Furchgott, G.M. Villani and D. Jothianandan, 1986, Phosphodiesterase inhibitors induce endothelium-dependent relaxation of rat and rabbit aorta by potentiating the effects of spontaneously released endothelium-derived relaxing factor. J. Pharmacol. Exp. Ther. 237:539-547

Maruyama, Y., 1987, A patch clamp study of mammalian platelets and their voltage-gated potassium current. J. Physiol. 391:467-485

Maurice, D.H. and R.J. Haslam, 1990a, Molecular basis of the synergistic inhibition of platelet function by nitrovasodilators and activators of adenylate cyclase: Inhibition of cyclic AMP breakdown by cyclic GMP. Mol. Pharmacol. 37:671-681

Maurice, D.H. and R.J. Haslam, 1990b, Nitroprusside enhances isoprenaline-induced increases in cAMP in rat aortic smooth muscle. Eur. J. Pharmacol. 191:471-475

Maurice, D.H., D. Crankshaw and R.J. Haslam, 1991, Synergistic actions of nitrovasodilators and isoprenaline on rat aortic smooth muscle. European J. Pharmacol. 192:235-242

May, D.C., E.M. Ross, A.G. Gilman and M.D. Smigel, 1985, Reconstitution of catecholamine-stimulated adenylate cyclase activity using three purified proteins. J. Biol. Chem. 260:15829-15833

McCall, T.B., N.K. Boughton-Smith, R.M.J. Palmer, B.J.R. Whittle and S. Moncada, 1989, Synthesis of nitric oxide from L-arginine by neutrophils: Release and interaction with superoxide anion. Biochem. J. 261:293-296

McKnight, G.S., C.H. Clegg, M.D. Uhler, J.C. Chrivia, G.G. Cadd, L.A. Correll and A.D. Otten, 1988, Analysis of the cAMP-dependent protein kinase system using molecular genetic approaches. Recent Progr. Hormone Res. 44:307-335

Mehta, J. and P. Mehta, 1980, Comparative effects of nitroprusside and nitroglycerin on platelet aggregation in patients with heart failure. J. Cardiovasc. Pharmacol. 2:25-33

Meisheri, K.D. and C. van Breeman, 1982, Effects of β-adrenergic stimulation on calcium movements in rabbit aortic smooth muscle:relationship with cyclic AMP. J. Physiol. 331:429-441

Meisheri, K.D., C.J. Taylor and H. Saneii, 1986, Synthetic atrial peptide inhibits intracellular calcium release in smooth muscle. Am. J. Physiol. 250:C171-C174

Mellion, B.T., L.J. Ignarro, E.H. Ohlstein, E.G. Pontecorvo, A.L. Hyman and P.J. Kadowitz, 1981, Evidence for the inhibitory role of guanosine 3',5'-monophosphate in ADP-induced human platelet aggregation in the presence of nitric oxide and related vasodilators. Blood 57:946-955

Miller, O.V. and R.R. Gorman, 1979, Evidence for distinct prostaglandin I_2 and D_2 receptors in human platelets. J. Pharmacol. Exp. Ther. 21:1134-140

Miot, F., P. Von Haastert and C. Erneux, 1985, Specificity of cGMP-binding to a purified cGMP-stimulated phosphodiesterase from bovine and renal tissue. Eur. J. Biochem. 149:59-65

Missiaen, L., I. Declerck, G. Droogmans, L. Plessers, H. DeSmedt, L. Raeymaekers and R. Casteels, 1989, Agonist-dependent Ca^{2+} and Mn^{2+} entry dependent on state of filling of Ca^{2+} stores in aortic smooth muscle of the rat. J. Physiol. 427:171-186

Mittal, C. K. and F. Murad, 1977, Formation of adenosine 3':5'-monophosphate by preparations of guanylate cyclase from rat liver and other tissues. J. Biol. Chem. 252:3136-3140

Moake, J.L., S.S. Tang, J.D. Olsen, J.H. Troll, O.L. Cimo and J.A. Davies, 1987, Platelets, von Willebrand factors and prostaglandin I_2. Am. J. Physiol. 241:H54-H59

Molina, R.C., J.W. Andersen, R.M. Rapoport, S. Waldman and F. Murad, 1987, Effects of in vivo nitroglycerin therapy on endothelium dependent and independent vascular relaxation and cyclic GMP accumulation in rat aorta. J. Cardiovasc. Pharmacol. 10:371-378

Moncada, S., 1982, Biological importance of prostacyclin. Br. J. Pharmacol. 76:3-31

Moncada, S. and J.R. Vane, 1979, The role of prostacyclin in vascular tissue. Fed. Proc. 38:66-71

Moore, J.B. and D.E. Schroechter, 1982, Purification and characterization of human lung calmodulin-independent cyclic AMP phosphodiesterase. Arch. Biochem. Biophys. 213:276-287

Morgan, J.P. and K.G. Morgan, 1982, Vascular smooth muscle: The first recorded Ca^{2+} transient. Pflügers Archiv. 395:75-77

Morgan, J.P. and K.G. Morgan, 1984, Alteration of cytoplasmic ionized calcium levels in smooth muscle by vasodilators in the ferret. J. Physiol. 357:539-551

Morgan, R.O. and A.C. Newby, 1989, Nitroprusside differentially inhibits ADP-stimulated calcium influx and mobilization in human platelets. Biochem. J. 258:447-454

Moritoki, H., T. Matsugi, H. Takase, H. Ueda and A. Tanioka, 1990, Evidence for the involvement of cyclic GMP in adenosine-induced, age-dependent vasodilation. Br. J. Pharmacol. 100:569-575

Muller, B., C. Lugnier and J.C. Stoclet, 1990, Implication of cyclic AMP in the positive inotropic effects of cyclic GMP-inhibited cyclic AMP phosphodiesterase inhibitors on guinea-pig isolated left atria. J. Cardiovasc. Pharmacol. 15:444-451

Murad, F., 1986, Cyclic guanosine monophosphate as a mediator of vasodilation. J. Clin. Invest. 78:1-9

Murray, R., L. Furci, and G.A. FitzGerald, 1989, Induction of prostacyclin receptor expression in human erythroleukemia cells. FEBS Lett. 258:172-174

Murphy, R.A., 1989, Contraction in smooth muscle cells. Ann. Rev. Physiol. 51:275-283

Murray, K.J., 1990, Cyclic AMP and mechanisms of vasodilation. Pharmac. Ther. 47:329-345

Murray, K.J., P.J. England, T.J. Hallam, T. Maguire, K. Moores, M.L. Reeves, A.W.M. Simpson and T.J. Rink, 1990, The effects of siguazodan, a selective phosphodiesterase inhibitor on human platelet function. Br. J. Pharmacol. 99:612-616

Myers, P.R., R.L. Minor, R. Guerra, J.N. Bates and D.G. Harrison, 1990, Vasorelaxant properties of the endothelium-derived relaxing factor more closely resemble S-nitrosocysteine than nitric oxide. Nature 345:161-163

Nabika, T., P.A. Velletri, T. Igawa, Y. Yamori and W. Lovenberg, 1985, Comparison of cyclic AMP accumulation and morphological changes induced by β-adrenergic stimulation of cultured vascular smooth muscle cells and fibroblasts. Blood Vessels 22:47-56

Nakamura, M., A. Nakamura, B. Fine and A. Aviv, 1988, Blunted cGMP response to ANF in vascular smooth muscle cells of SHR. Am. J. Physiol. 255:C573-C580

Nakane, M., S. Saheki, T. Kuno, K. Ishii and F. Murad, 1988, Molecular cloning of a cDNA coding for 70 kilodalton subunit of soluble guanylate cyclase from rat lung. Biochem. Biophys. Res. Commun. 157:1139-1147

Nakane, M., K. Arai, S. Saheki, T. Kuno, W. Buechler and F. Murad, 1990, Molecular cloning and expression of cDNA coding for soluble guanylate cyclase from rat lung. J. Biol. Chem. 265:16841-16845

Nakashima, S., T. Tohmatsu, H. Hattori, Y. Okano and Y. Nozawa, 1986, Inhibitory action of cyclic GMP on secretion, polyphosphoinositide hydrolysis and calcium mobilization in thrombin-stimulated human platelets. Biochem. Biophys. Res. Commun. 135:1099-1104

Nakatsu, K. and J. Diamond, 1989, Role of cGMP in relaxation of vascular and other smooth muscle. Can. J. Physiol. Pharmacol. 67:251-262

Nemoz, G., M. Moueqquit, A.-F. Prigent and H. Pacheco, 1989, Isolation of similar rolipram-inhibitible cyclic-AMP-specific phosphodiesterases from rat brain and heart. Eur. J. Biochem. 184:511-520.

Nesbitt, J.A., W.B. Anderson, Z. Miller, I. Pastan, T.R. Russell and D. Gospodarowicz, 1976, Guanylate cyclase and cyclic 3':5'-monophosphate phosphodiesterase activities and cyclic guanosine 3':5'-monophosphate levels in normal and transformed fibroblast culture. J. Biol. Chem. 251:2344-2352

Nielson, C.P. and R.E. Vestal, 1989, Effects of adenosine on polymorphonuclear leucocyte function, cyclic 3':5'-adenosine monophosphate, and intracellular calcium. Br. J. Pharmacol. 97:882-888

Nishikawa, M., M. Kanamori and H. Hidaka, 1982, Inhibition of platelet aggregation and stimulation of guanylate cyclase by an antianginal agent molsidomine and its metabolites. J. Pharmacol. Exp. Ther. 220:183-189

Nishimura, J. and C. van Breeman, 1989, Direct regulation of smooth muscle contractile elements by second messengers. Biochem. Biophys. Res. Commun. 163:929-935

Nishizuka, Y., 1984, The role of protein kinase C in cell surface signal transduction and tumour promotion. Nature 308:693-698

Ogreid, D., S.O. Doskeland and J.P. Miller, 1983, Evidence that cyclic nucleotides activating rabbit muscle protein kinase I interact with both types of cAMP binding sites associated with the enzyme. J. Biol. Chem. 258:1041-1049

Ohuchi, S., H. Hagiwara, M. Ishido, T. Fujita, T. Kume, I. Ishigaki and S. Hirose, 1989, Mechanism of activation of particulate gyanylate cyclase by atrial natriuretic peptide as deduced from radiation inactivation analysis. Biochem. Biophys. Res. Comm. 158:603-609

O'Rourke, R., G.B. Zavoico, L.H. Smith and M.B. Feinstein, 1987, Stimulus response-coupling in a cell free platelet membrane system. FEBS Lett. 214:176-180

O'Rourke, F. G.B. Zavoico and M.B. Feinstein, 1989, Release of Ca^{2+} by inositol 1,4,5-trisphosphate in platelet membrane vesicles is not dependent on cyclic cAMP-dependent protein kinase. Biochem. J. 257:715-721

Ostermann, G., U. Till and K. Thielman, 1983, Studies on the stimulation of human blood platelets by semi-synthetic platelet-activating factor. Thromb. Res. 30:127-136

Ousterhaut, J.M. and N. Sperelakis, 1987, Cyclic nucleotides depress action potentials in cultured aortic smooth muscle cells. Eur. J. Pharmacol. 144:7-14

Paglin, S., Y. Takuwa, K.E. Kamm, J.T. Stull, H. Gavras and H. Rasmussen, 1988, Atrial natriuretic peptide inhibits the agonist-induced increase in extent of myosin light chain phosphorylation in aortic smooth muscle. J. Biol. Chem. 263:13117-13120

Palmer, R.M.J., A.G. Ferrige and S. Moncada, 1987, Nitric oxide release accounts for the biological activity of endothelium-derived relaxing factor. Nature 327:524-526

Palmer, R.M.J., D.S. Ashton and S. Moncada, 1988, Vascular endothelial cells synthesize nitric oxide from L-arginine. Nature 333:664-666

Parks, T.P., A.C. Nairn, P. Greengard and J.D. Jamieson, 1987, The cyclic nucleotide-dependent phosphorylation of aortic smooth muscle membrane proteins. Arch. Biochem. Biophys. 255:361-371

Paul, D.K., R.B. Marala, R.K. Jaiswal and R.K. Sharma, 1987, Coexistence of guanylate cyclase and atrial natriuretic factor receptor in a 180 kDa protein. Science 235:1224-1226

Pöch, G. and S. Holzmann, 1980, Quantitative estimation of overadditive and underadditive drug effects by means of theoretical, additive dose-response curves. J. Pharmacol. Methods 4:179-188

Polakis, P.G., R.F. Weber, B. Nevina, J.R. Didsbury, T. Evans and R. Snyderman, 1989, Identification of the *ral* and *rac1* gene products, low molecular mass GTP-binding proteins from human platelets. J. Biol. Chem. 264:16383-16389

Popescu, L.M., Panoiu, M. Hinescu and O. Natu, 1985, The mechanism of cGMP-induced relaxation in vascular smooth muscle. Eur. J. Pharmacol. 107:393-394

Pyne, N.J., N. Anderson, B.E. Lavan, G. Milligan, H.G. Nimmo and M.D. Houslay, 1987a, Specific antibodies and the selective inhibitor ICI 778233 demonstrate that the hormonally stimulated 'dense-vesicle' and peripheral plasma membrane cyclic AMP phosphodiesterase display distinct tissue distributions in the rat. Biochem. J. 248:897-901.

Pyne, N.J., M.E. Cooper and M.D. Houslay, 1987b, The insulin- and glucagon-stimulated 'dense-vesicle' high affinity cyclic AMP phosphodiesterase from rat liver. Biochem. J. 249:33-42

Radomski, M.W., R.M.J. Palmer and S. Moncada, 1987a, Comparative pharmacology of endothelium-derived relaxing factor, nitric oxide and prostacyclin. Br. J. Pharmacol. 92:181-187

Radomski, M.W., R.M.J. Palmer and S. Moncada, 1987b, The anti-aggregating properties of vascular endothelium: interactions between prostacyclin and nitric oxide. Br. J. Pharmacol. 92:639-646

Radomski, M.W., R.M.J. Palmer and S Moncada, 1990a, An L-arginine to nitric oxide pathway in human platelets regulates aggregation. Proc. Natl. Acad. Sci. U.S.A. 87:5193-5197

Radomski, M.W., R.M.J. Palmer and S. Moncada, 1990b, Characterization of the L-arginine: nitric oxide pathway in human platelets. Br. J. Pharmacol. 101:325-328

Raeymaekers, L., F. Hofmann and R. Casteels, 1988, Cyclic GMP-dependent protein kinase phosphorylation of phospholamban in isolated sarcoplasmic reticulum from cardiac and smooth muscle. Biochem. J. 252:269-273

Rao, G.H.R., J.G. White and C.A. Cox, 1987, Influence of a calcium-dependent protease inhibitor on platelet activation and secretion. Thromb. Res. 47:625-637

Randerath, K. and E. Randerath, 1967, Thin layer separation methods for nucleic acid derivatives. Methods Enzymol 12:323-347

Rangel-Aldao, R. and O.M. Rosen, 1976, Dissociation and reassociation of the phosphorylated and nonphosphorylated forms of adenosine 3':5'-monophosphate-dependent protein kinase from bovine cardiac muscle. J. Biol. Chem. 251:3375-3380

Rapoport, R.M., 1986, Cyclic guanosine monophosphate inhibition of contraction may be mediated through inhibition of phosphatidylinositol hydrolysis in rat aorta. Circulation Res. 58:407-410

Rapoport, R.M. and F. Murad, 1983, Agonist-induced endothelium-dependent relaxation in rat thoracic aorta may be mediated through cGMP. Circulation. Res. 52:352-357

Rapoport, R.M., M.B. Draznin and F. Murad, 1982, Sodium nitroprusside induced protein phosphorylation in intact rat aorta is inhibited by 8 Br cGMP. Proc. Natl. Acad. Sci. U.S.A. 79:6470-6474

Rapoport, R.M., M.B. Draznin and F. Murad, 1983, Endothelium-dependent relaxation in rat aorta may be mediated through cyclic GMP-dependent protein phosphorylation. Nature 306:174-176

Rascon, A., P. Belfrage, E. Degerman, S. Lindgren, K.E. Andersson, A. Newman, V.C. Manganiello, and L. Stavenow, 1989, Purification of a cGMP-inhibited cAMP phosphodiesterase from vascular smooth muscle in: "Purine Nucleosides and Nucleotides in Cell Signalling Targets for New Drugs", Eds. Jacobsen, K; J. Daly, and V.C. Manganiello, pp. 358-364, Springer-Verlag, New York, .

Rashatwar, S.S., T.L. Cornwell and T.M. Lincoln, 1987, Effects of 8-bromo-cGMP on Ca^{2+} levels in vascular smooth muscle cells: Possible regulation of Ca^{2+}-ATPase by cGMP-dependent protein kinase. Proc. Natl. Acad. Sci. U.S.A. 84:5685-5689

Rasmussen, H., Y. Takuwa and S. Park, 1987, Protein kinase C in the regulation of smooth muscle contraction. FASEB J. 1:177-185

Resink, T.J., T. Scott-Burdon, C.R. Jones, U. Baur and F.R. Buhler, Atrial natriuretic peptide: binding and cyclic GMP response in cultured vascular smooth muscle cells from spontaneously hypertensive rats. Am. J. Hypertension 2:32-39

Rhodin, J.A., 1980, Architecture of the vessel wall, in "Handbook in Physiology: The Cardiovascular System, Vol II.Eds., Bohr, D.F., A.P. Somlyo and H.V. Sparks, pp.1-31, Elsevier, Amsterdam

Rhee, S.G., P.-G Suh, S.-H Ryu and S.Y. Lee, 1989, Studies of inositol phospholipid-specific phospholipase C. Science 244:546-550

Rimele, T.J., R.J. Sturm, L.M. Adams, D.E. Henry, R.J. Heaslip, B.M. Weichman and D. Grimes, 1988, Interaction of neutrophils with vascular smooth muscle: Identification of a neutrophil-derived relaxing factor. J. Pharmacol. Exp. Ther. 245:102-111

Rink, T.J. and S.O. Sage, 1990, Calcium signalling in human platelets. Ann. Rev. Physiol. 52:431-449

Rink, T.J., S.W. Smith and R.Y. Tsien, 1982, Cytoplasmic free Ca^{2+} in human platelets: Ca^{2+} thresholds and Ca-independent activation for shape-change and secretion. FEBS Lett. 148:21-26

Rittenhouse-Simmons, S., 1979, Production of diglyceride from phosphatidylinositol in activated human platelets. J. Clin. Invest. 63:580-587

Ross, E.M. and A.G. Gilman, 1980, Biochemical properties of hormone-sensitive adenylate cyclase. Ann. Rev. Biochem. 49:533-564

Rubanyi, G. and P.M. Vanhoutte, 1985, Endothelium-removal decreases relaxations of canine coronary arteries caused by β-adrenergic agonists and adenosine. J. Cardiovasc. Pharmacol. 7:139-144.

Rüegg, J.D., 1986, Calcium in Muscle Activation: A Comparative Approach, pp. 155-164, Springer-Verlag, Berlin

Sadoshima, J.-I., N. Akaike, H. Kanaide and M. Nakamura, 1988, Cyclic AMP modulates Ca-activated K channel in cultured smooth muscle cells of rat aortas. Am. J. Physiol. 255:H754-H759

Sage, S.O. and T.J. Rink, 1986, Effects of ionic substitution on $[Ca^{2+}]i$ rise evoked by thrombin and PAF in human platelets. Eur. J. Pharmacol. 128:99-107

Sage, S.O., J.E. Merritt, T.J. Hallam and T.J. Rink, 1989, Receptor-mediated Ca^{2+} entry in fura-2 loaded human platelets stimulated with ADP and thrombin. Biochem. J. 258:923-926

Sage, S.O., R. Raest and T.J. Rink, 1990, ADP evokes biphasic Ca^{2+} influx in fura-2-loaded human platelets: Evidence for Ca^{2+} entry regulated by the intracellular Ca^{2+} store. Biochem. J. 265:675-680

Saida, K. and C. van Breeman, 1984, Cyclic AMP modulation of adrenoreceptor-mediated arterial smooth muscle contraction. J. Gen. Physiol. 84:307-318

Saida, K. and C. van Breeman, 1987, GTP requirement for inositol-1,4,5-trisphosphate-induced Ca^{2+} release from sarcoplasmic reticulum in smooth muscle. Biochem. Biophys. Res. Commun. 144:1313-1316

Saida, K., C. Twort and C. van Breeman, 1988, The specific GTP requirement for inositol 1,4,5-trisphosphate-induced Ca^{2+} release from skinned vascular smooth muscle. J. Cardiovasc. Pharmacol. 12:547-550

Salama, S.E. and R.J. Haslam, 1984, Characterization of the protein kinase activities of human platelet supernatant and particulate fractions. Biochem. J. 218:285-294

Sands, H., D. Sinclair and J. Mascali, 1976, Cyclic AMP and protein kinase in the spontaneously hypertensive rat aorta and tissue-cultured aortic smooth muscle cells. Blood Vessels 13:361-373

Saxon, A., and H.E. Kattlove, 1976, Platelet inhibition by sodium nitroprusside, a smooth muscle inhibitor. Blood 47:957-961

Schafer, A.I., B. Cooper, D. O'Hara and R.I. Handin, 1979, Identification of platelet receptors for prostaglandin I_2 and D_2. J. Biol. Chem. 254:2914-2917

Schatzmann, H.J., 1989, The calcium pump of the surface membrane and the sarcoplasmic reticulum. Ann. Rev. Physiol. 51:473-485

Schillinger, E. and G. Prior, 1980, Prostaglandin I_2 receptors in a particulate fraction of platelets and various species. Biochem. Pharmacol. 29:2297-2299

Schimmerlik, M.I., 1989, Structure and regulation of muscarinic receptors. Ann. Rev. Physiol. 51:217-227.

Schini, V. and P.M. Vanhoutte, 1991, L-arginine evokes both endothelium-dependent and -independent relaxations in L-arginine-depleted aortas of the rat. Circulation Res. 68:209-216

Schoeffter, P. and J.-C. Stoclet, 1982, Age-related decrease of in vitro isoproterenol-induced cyclic AMP accumulation in rat aorta. Eur. J. Pharmacol. 77:183-186

Schoeffter, P., C. Lugnier, F. Demesy-Waeldele and J.C. Stoclet, 1987, Role of cyclic AMP- and cyclic GMP-phosphodiesterases in the control of cyclic nucleotide levels and smooth muscle tone in rat isolated aorta. A study with selective inhibitors. Biochem. Pharmacol. 36:3965-3972

Schrör, K., H. Darius, R. Matzky and R. Ohlendorf, 1981, The antiplatelet and cardiovascular actions of a new carbacyclin derivative (ZK 36374) - Equipotent to PGI_2 in vitro. Naunyn-Schmiedeberg's Arch. Pharmacol. 316:252-257

Schultz, G., J.G. Hardman, K. Schultz, C.E. Baird and E.W. Sutherland, 1973, The importance of calcium ions for the regulation of guanosine 3',5'-monophosphate levels. Proc. Natl. Acad. Sci. U.S.A. 77:5537-5541

Schulz, S., M. Chinkers and D.L. Garbers, 1989, The guanylate cyclase/receptor family of proteins. FASEB J. 3:2026-2035

Seamon, K.B. and J.W. Daly, 1981, Forskolin: A unique diterpine activator of cyclic AMP-generating systems. J. Cyclic Nucleotide Res. 7:201-224

Sellers, J.R. and R.S. Adelstein, 1987, Regulation of contractile activity. The Enzymes 17B:381-418

Senter, P.D., F. Eckstein, A. Mülsch and E. Böhme, 1983, The stereochemical course of the reaction catalyzed by soluble bovine lung guanylate cyclase. J. Biol. Chem. 258:6741-6745

Shabb, J.B., L. Ng and J.D. Corbin, 1990, One amino acid change produces a high affinity cGMP-binding site in cAMP-dependent protein kinase. J. Biol. Chem. 265:16031-16034

Shaji, S., L.H.J. Ericsson, K.A. Walsh, E.H. Fisher and K. Titani, 1983, Amino acid sequence of the catalytic subunit of bovine type II adenosine cyclic 3',5'-phosphate dependent protein kinase. Biochemistry 22:3702-3709

Sharma, R.K. and J.H. Wang, 1986, Purification and characterization of bovine lung calmodulin-dependent cyclic nucleotide phosphodiesterase: An enzyme containing calmodulin as a subunit. J. Biol. Chem. 261:14160-14166

Sharma, R.K., T.H. Wang, E. Wirch and J.H. Wang, 1980, Purification and properties of bovine brain calmodulin-dependent cyclic nucleotide phosphodiesterase. J. Biol. Chem. 255:5916-5923

Shimokawa, H., N.A. Flavahan, R.R. Lorenz and P.M. Vanhoutte, 1988, Prostacyclin releases endothelium-derived relaxing factor and potentiates its action in coronary arteries of the pig. Br. J. Pharmacol. 95:1197-1203

Shimomura, H., L.J. Dangott, D.L. Garbers, 1986, Covalent coupling of a resact analogue to guanylate cyclase. J. Biol. Chem. 261:15778-15782

Siegel, A.M., J.B. Smith, M.J. Silver, K.C. Nicolaou and D. Ahein, 1979, Selective binding site for [^{3}H]prostacyclin on platelets. J. Clin. Invest. 63:215-220

Siess, W., 1989, Molecular mechanisms of platelet activation. Physiol. Rev. 69:58-178

Siess, W. and E.G. Lapetina, 1990, Functional relationship between cyclic AMP-dependent protein phosphorylation and platelet inhibition. Biochem. J. 271:815-819

Silk, S.T., S. Clejan and K. Witkom, 1989, Evidence of GTP-binding protein regulation of phospholipase A_2 activity in isolated human platelet membranes. J. Biol. Chem. 264:21466-21469

Silver, P.J., 1985, Regulation of contractile activity in vascular smooth muscle by protein kinases. Rev. Clin. Basic Pharm. 5:341-395

Silver, P.J., R.E. Lepore, B. O'Connor, B.M. Lemp, L.T. Hamel, R.G. Bentley and A.L. Harris, 1988, Inhibition of the low K_m cyclic AMP phosphodiesterase and activation of the cyclic AMP system in vascular smooth muscle by milrinone. J. Pharmacol. Exp. Ther. 247:34-42

Silver, P.J., A.L. Harris, R.A. Buchholz, M.S. Miller, R.J. Gordon, R.L. Dundore and E.D. Pagani, 1989, Low K_m cAMP phosphodiesterase isozymes and modulation of tone in vascular, airway and gastrointestinal smooth muscle, in Purine Nucleosides and Nucleotides in Cell Signalling: Targets for New Drugs, Eds. Jacobsen, K., J. Daly, and V.C. Manganiello, pp. 358-364, Springer-Verlag, New York

Simon, M.F., H. Chap, and L. Douste-Blazy, 1988, Effects of a stimulant of guanylyl cyclase, SIN-1, on calcium movements and phospholipase C activation in thrombin-stimulated human platelets. Biochem. Pharmacol. 37:1263-1269

Simonds, W.F., P.K. Goldsmith, J. Codina, C.G. Unson and A.M. Spiegel, 1989, G_i2 mediates α2-adrenergic inhibition of adenylyl cyclase in platelet membranes: in situ identification with G_α C-terminal antibodies. Proc. Natl. Acad. Sci. U.S.A. 86:7809-7813

Simpson, A.W.H., M.I. Reeves and T.J. Rink, 1988, Effects of SK&F 94120, an inhibitor of cyclic nucleotide phosphodiesterases type III, on human platelets. Biochem. Pharmacol. 37:2315-2320

Smigel, M.D., 1986, Purification of the catalyst of adenylate cyclase. J. Biol. Chem. 261:1976-1982

Smith, J.B., L. Smith, E.R. Brown, D. Barns, M.A. Sabir, J.S. Davis and R.V. Farese, 1984, Angiotensin II rapidly increases phosphatidate-phosphoinositol synthesis and phosphoinositide hydrolysis and mobilizes intacellular Ca^{2+} in cultured arterial muscle cells. Proc. Natl. Acad. Sci. U.S.A. 87:7812-7816

Sobieszek, A., 1977, Vertebrate smooth muscle myosin. Enzymatic and structural properties in: "The Biochemistry of Smooth Muscle", Ed. N.L. Stephens, pp. 413-443, Univ. Press, Baltimore

Somlyo, A.P., 1980, Ultrastructure of vascular smooth muscle, in "Handbook of Physiology: The cardiovascular system, Vol. II" Eds. Böhr, D.F., A.P. Somlyo, H.V. Sparks, pp.33-67, Elsevier, Amsterdam

Somlyo, A.P. and A.V. Somlyo, 1990, Flash photolysis studies of excitation-contraction coupling, regulation and contraction in smooth muscle. Ann. Rev. Physiol. 52:857-874

Souness, J.E., R. Brazdil, B.K. Diocee and R. Jordan, 1989, Role of selective cyclic GMP phosphodiesterase inhibition in the myorelaxant actions of M&B 22,948, MY-5445, vinpocetine and 1-methyl-3-isobutyl-8-(methylamino)xanthine. Br. J. Pharmacol. 98:725-734

Spiegel, A.M., 1989, Signal transduction by guanine nucleotide binding proteins. Mol. Cell. Endocrinol. 49:1-16

Stamler, J.S., D.E. Vaughan and J. Loscalzo, 1989, Synergistic dissaggregation of platelets by tissue plasminogen activator, prostaglandin E_1, and nitroglycerin. Circulation Res. 65:796-804

Sudoh, T., K. Kangawa, N. Minamino and H. Matsuo, 1988, A new natriuretic peptide in porcine brain. Nature 332:78-81

Sudoh, T., N. Minamino, K. Kangawa and H. Matsuo, 1990, C-type natriuretic peptide (CNP): A new member of natriuretic peptide family identified in porcine brain. Biochem. Biophys. Res. Commun. 168:863-870

Suematsu, E., M. Hirata, T. Hashimoto and H. Kuriyama, 1984, Inositol 1,4,5-trisphosphate releases Ca^{2+} from intracellular store sites in skinned single cells of porcine coronary artery. Biochem. Biophys. Res. Commun. 120:481-485

Supattapone, S., S.K. Danoff, A. Theibert, S.K. Joseph, J. Steiner and S.H. Snyder, 1988, Cyclic AMP-dependent phosphorylation of a brain inositol trisphosphate receptor decreases its release of calcium. Proc. Natl. Acad. Sci. U.S.A. 85:8747-8750

Suthi, K.S. and N. Crawford, 1985, Inositol 1,4,5-trisphosphate release of sequestered Ca^{2+} from highly purified human platelet intracellular membranes. Biochem. J. 230:247-253

Sweat, J.D., T.M. Connolly, E.J. Cragoe and L.E. Limbird, 1986, Evidence that Na^+/H^+ exchange regulates receptor-mediated phospholipase A_2 activation in human platelets. J. Biol. Chem. 261:8667-8673

Takai, Y., K. Kaibuchi, T. Matsubara and Y. Nishizuka, 1981, Inhibitory action of guanosine 3',5'-monophosphate on thrombin-induced phosphatidylinositol turnover and protein phosphorylation in human platelets. Biochem. Biophys. Res. Commun. 101:61-67

Takio, K., R.D. Wade, S.B. Smith, E.G. Krebs, K.A. Walsh and K. Titani, 1984, Guanosine cyclic 3',5'-phosphate dependent protein kinase, a chimeric protein homologous with two separate protein families. Biochemistry 23:4207-4218

Takuwa, Y., G. Kelley, N. Takuwa and H. Rasmussen, 1988a, Protein phosphorylation changes in bovine carotid artery smooth muscle during contraction and relaxation. Mol. Cell. Endorinology. 60:71-86

Takuwa, Y., N. Takuwa and H. Rasmussen, 1988b, The effects of isoproterenol on intracellular calcium concentration. J. Biol. Chem. 263:762-768

Tamamoto, K., N. Yamamoto, H. Kitagawa, K. Tanone, G. Kosaki and H. Yamazaki, 1986, Localization of a thrombin-binding site on human platelet membranes: glycoprotein Ib determined by a monoclonal antibody. Thromb. Haemostas. 55:162-167

Tanaka, T., T. Ishikawa, M. Hagiwara, K. Onada, H. Itoh and H. Hidaka, 1988, Effects of cilostazol, a selective cAMP phosphodiesterase inhibitor, on the contraction of vascular smooth muscle. Pharmacology 36:3130-320

Tanaka, T., H. Ohta, K. Kanada, T. Tanaka, H. Hidaka and K. Sobue, 1990, Phosphorylation of high-M_r caldesmon by protein kinase C modulates the regulatory function of this protein on the interaction between actin and myosin. Eur. J. Biochem. 188:495-500

Tateson, J.E., S. Moncada and J.R. Vane, 1977, Effects of prostacyclin (PGX) on cyclic AMP concentrations in human platelets, Prostaglandins 13:389-394

Taylor, C.J. and K.D. Meisheri, 1986, Inhibitory effects of a synthetic atrial peptide on contraction and ^{45}Ca fluxes in vascular smooth muscle. J. Pharmacol. Exp. Ther. 237:803-808

Thomas, M.K., S.H. Francis and J.D. Corbin, 1990, Substrate and kinase-directed regulation of phosphorylation of a cGMP-binding phosphodiesterase by cGMP. J. Biol. Chem. 265:14971-14978.

Thompson, W.J. and M.M. Appleman, 1971, Multiple cyclic nucleotide phosphodiesterase activities from rat brain. Biochemistry 10:311-316

Thompson, W.J., P.W. Epstein, and S.J. Strada, 1979, Purification and characterization of a high affinity cyclic adenosine monophosphate phosphodiesterase from dog kidney. Biochemistry 18:5228-5237.

Thorpe, D.S. and D.L. Garbers, 1989, The membrane form of guanylate cyclase: Homology with a subunit of the cytoplasmic form of the enzyme. J. Biol. Chem. 264:6545-6549

Torphy, T.J. and L.B. Cieslinski, 1990, Characterization and selective inhibition of cyclic nucleotide phosphodiesterase isozymes in canine tracheal smooth muscle. Mol. Pharmacol. 37:206-211

Torphy, T.J., Zheng, C., S.M. Peterson, R.R. Fiscus, G.A. Rinard and S.E. Mayer, 1985, Inhibitory effect of metacholine on drug-induced relaxation, cyclic AMP accumulation, and cyclic AMP-dependent protein kinase activation in canine tracheal smooth muscle. J. Pharmacol. Exp. Ther. 233:409-417

Tremblay, J., R. Gerzer and P. Hamet, 1988, Cyclic GMP in cell function, Adv. Sec. Mess. Phosphoprotein Res. 22:319-383.

Trong, H., W. Sonnenberg, N. Beier, H. Charbonneau, J.A. Beavo and K. Walsh, 1990, Amino acid sequence of the cyclic GMP-stimulated cyclic nucleotide phosphodiesterase from bovine heart. Biochemistry 29:10280-10288

Tsai, S.-C, V.C. Manganiello and M. Vaughan, 1978, Purification of guanylate cyclase from rat liver supernatant. J. Biol. Chem. 253:8452-8457

Tsai, A., M.J. Hsu, H.. Vijjeswarapu and K.K. Wu, 1989, Solubilization of prostacyclin membrane receptor from human platelets. J. Biol. Chem. 264:61-67

Tuteja, N., M. Donciges, I. Klisak, R. Tuteja, G. Inana, T. Mohandas, T.S. Sparkes and D.B. Forbes, 1990, Isolation and characterization of cDNA encoding the gamma-subunit of cGMP phosphodiesterase in human retina. Gene 88:227-232

Tyers, M., R.A. Rachubinski, M.I. Stewart, P.M. Varrichio, R.G.L. Shorr, R.J. Haslam and C.B. Harley, 1988, Molecular cloning and expression of the major protein kinase substrate of platelets. Nature 333:470-473

Twort, C.H.C. and C. van Breeman, 1988, Cyclic guanosine monophosphate-enhanced sequestration of Ca^{2+} by sarcoplasmic reticulum in vascular smooth muscle. Circulation Res. 62:961-964

Vallet, B., A. Molla and J.G. Demaille, 1981, Cyclic adenosine monophosphate dependent regulation of purified bovine aortic calcium/calmodulin-dependent myosin light chain kinase. Biochim. Biophys. Acta 674:256-264

van Breeman, C. and K. Saida, 1989, Cellular mechanisms regulating $[Ca^{2+}]i$ in smooth muscle. Ann. Rev. Physiol. 51:315-329

Varsanyi, M., H.-G. Tölle, L.M.G. Heilmeyer, R.M.C. Dawson and R.F. Irvine, 1983, Activation of sarcoplasmic reticulum Ca^{2+} transport ATPase by phosphorylation of an associated phosphatidylinositol. EMBO. J. 2:1543-1548

Verghese, M.W. and C. Frangakis, 1988, Milrinone, a novel cAMP phosphodiesterase inhibitor used to treat congestive heart failure inhibits human neutrophil function. Clin. Res. 36:A474-A474

Verhaeghe, R.H., 1977, Action of adenosine and adenine nucleotides on dog's isolated veins. Am. J. Physiol. 233:H114-H121

Vrolix, M., L. Raeymaekers, F. Wuytack, F. Hofmann and R. Casteels, 1988, Cyclic GMP-dependent protein kinase stimulates the plasmalemmal Ca^{2+} pump of smooth muscle via phosphorylation of phosphatidylinositol. Biochem. J. 255:855-863

Wada, H., V.C. Manganiello and J.C. Osborne, 1987, Analysis of the kinetics of cyclic AMP hydrolysis by the cyclic GMP-stimulated cyclic nucleotide phosphodiesterase. J. Biol. Chem. 262:13938-13945

Waldman, S.A. and F. Murad, 1987, Cyclic GMP synthesis and function. Pharmacol. Rev. 39:163-196

Waldman, S.A., J.A. Lewicki, H.J. Brandwein and F. Murad, 1982, Partial purification and characterization of particulate guanylate cyclase from rat liver after solubilization with trypsin. J. Cyclic Nucleotide Res. 8:359-370.

Waldman, S.A., J.A. Lewicki, L.Y. Chang and F. Murad, 1983, Highly purified particulate guanylate cyclase from rat lung: characterization and comparison with soluble guanylate cyclase. Mol. Cell. Biochem. 57:155-166.

Waldman, S.A., R.M. Rapoport and F. Murad, 1984, Atrial natriuretic factor selectively activates particulate guanylate cyclase and elevates cyclic GMP in rat tissues. J. Biol. Chem. 259:14332-14334

Waldmann, R. and U. Walter, 1989, Cyclic nucleotide elevating vasodilators inhibit platelet aggregation at an early step of the activation cascade. Eur. J. Pharmacol. 159: 317-320

Waldmann, R., S. Bauer, C. Gobel, F. Hofmann, K.H. Jakobs and U. Walter, 1986, Demonstration of cGMP-dependent protein kinase and cGMP-dependent phosphorylation in cell-free extracts of platelets. Eur. J. Biochem. 158:203-210

Waldmann, R., M. Neiberding and U. Walter, 1987, Vasodilator-stimulated protein phosphorylation in platelets is mediated by cAMP- and cGMP-dependent protein kinases. Eur. J. Biochem. 167:441-448

Walter, U., 1989, Physiological role of cGMP and cGMP-dependent protein kinase in the cardiovascular system. Rev. Physiol. Biochem. Pharmacol. 113:42-88

Wang, J.H., R.J. Sharma and M.J. Mooibroek, 1990, Calmodulin stimulated cyclic nucleotide phosphodiesterase in "Cyclic nucleotide Phosphodiesterases: Structure, Regulation and Drug Action, Eds. Beavo, J. and M.D. Houslay, pp. 19-59, Wiley, Chichester

Wardell, M.R., C.C. Reynolds, M.C. Berndt, R.W. Wallace and J.E.B. Fox, 1989, Platelet glycoprotein Ibβ is phosphorylated on serine 166 by cyclic AMP-dependent protein kinase. J. Biol. Chem. 264:15656-15661

Watras, J., 1988, Regulation of calcium uptake in bovine aortic sarcoplasmic reticulum by cAMP-dependent protein kinase. J. Mol. Cell Cardiol. 20:711-723

Weber, H.W. and M.M. Appleman, 1982, Insulin-dependent and insulin-independent low K_m cyclic AMP phosphodiesterase from rat adipose tissue. J. Biol. Chem. 257:5339-5341

Weber, I.T., J.B. Shabb and J.D. Corbin, 1989, Predicted structures of the cGMP-binding domain of the cGMP-dependent protein kinase: A key alanine/threonine difference in evaluating any divergence of cAMP and cGMP-binding sites. Biochemistry 28:6122-6127.

Weishaar, R.E., M.H. Cain and J.A. Bristol, 1985, Multiple molecular forms of phosphodiesterase and the potential for drug selectivity, J. Med. Chem. 28:537-545.

Weishaar, R.E., S.D. Burrows, D.C. Kobylarz, M.M. Quade and D.B. Evans, 1986, Multiple molecular forms of cyclic nucleotide phosphodiesterase in cardiac and smooth muscle and in platelets: Isolation, characterization, and effects of various reference phosphodiesterase inhibitors and cardiotonic agents. Biochem. Pharmacol. 35:787-800

Weishaar, R.E., D.C. Kobylarz-Singer and H.R. Kaplan, 1987a, Subclasses of cyclic AMP phosphodiesterase in cardiac muscle. J. Mol. Cell Cardiol. 19:1025-1036.

Weishaar, R.E., D.C. Kobylarz-Singer, R.P. Steffen and H.R. Kaplan, 1987b, Subclasses of cyclic AMP-specific phosphodiesterase in left ventricular muscle and their involvement in regulating myocardial contractility. Circulation. Res. 61:539-547.

Weishaar, R.E., D.C. Kobylarz-Singer, J. Keiser, S.J. Haleen, T.C. Major, S. Rapundalo, J.T. Peterson and R. Panek, 1990, Subclasses of cyclic GMP-specific phosphodiesterase and their role in regulating the effects of atrial natriuretic factor. Hypertension 15:528-540.

White, J.G., 1987, An overview of platelet structural physiology. Scan. Microscopy 1:1677-1700

White, T.E., J.-C. Lacal, B. Reep, T.H. Fischer, E.G. Lapetina and G.C. White, 1990, Thrombolamban, the 22 kDa platelet substrate of cyclic AMP-dependent protein kinase, is immunologically homologous with the Ras family of GTP-binding proteins. Proc. Natl. Acad. Sci. U.S.A. 87:758-762

Williams, M., 1987, Purine receptors in mammalian tissues: Pharmacology and functional significance. Ann. Rev. Pharmacol. Toxicol. 27:315-345

Williams, K.A., W. Murphy and R.J. Haslam, 1987, Effects of activation of protein kinase C on the agonist-induced stimulation and inhibition of cyclic AMP formation in intact human platelets. Biochem. J. 243:667-678

Willis, A.L., D.L. Smith, M. Loveday, J. Fulks, C.H. Lee, L. Hedley and D. VanAntwerp, 1989, Selective anti-platelet aggregation synergism between a prostacyclin-mimetic, RS93427 and the nitrodilators sodium nitroprusside and glyceryl trinitrate. Br. J. Pharmacol. 98:1296-1303

Winder, S.J. and M.P. Walsh, 1990, Smooth muscle calponin: inhibition of actomyosin MgATPase and regulation by phosphorylation. J. Biol. Chem. 265:10148-10155

Wolfe, L., J.D. Corbin and S.H. Francis, 1989a, Characterization of a novel isozyme of cGMP-dependent protein kinase from bovine aorta. J. Biol. Chem. 264:7734-7741

Wolfe, L., S.H. Francis and J.D. Corbin, 1989b, Properties of a cGMP-dependent monomeric protein kinase from bovine aorta. J. Biol. Chem. 264:4157-4162

Wolff, J., C. Londos, and D.M.F. Cooper, 1981, Adenosine receptors and the regulation of adenylate cyclase. Adv. Cyclic Nucleotide Res. 14:199-214

Wood, K.S., G.M. Buga, R.E. Byrns and L.J. Ignarro, 1990, Vascular smooth muscle-derived relaxing factor (MDRF) and its close similarity to nitric oxide. Biochem. Biophys. Res. Commun. 170:80-88

Wright, C.D., P.J. Kuipers, D. Kobylarz-Singer, L.J. Devall, B.A. Klinkefus and R.E. Weishaar, 1990, Differential inhibition of human neutrophil functions: Role of cyclic AMP-specific, cyclic GMP-insensitive phosphodiesterase. Biochem. Pharmacol. 40:699-707

Yada, Y., S. Nagao, Y. Okana and Y. Nozawa, 1989, Inhibition by cyclic AMP of guanine nucleotide-induced activation of phosphoinositide-specific phospholipase C in human platelets. FEBS Lett. 242:368-372

Yamamoto, T., F. Lieberman, J.C. Osborne, V.C. Manganiello, M. Vaughan and H. Hidaka, 1984, Selective inhibition of two soluble adenosine cyclic 3':5'-phosphate phosphodiesterases partially purified from calf liver. Biochemistry 23:670-675

Yatani, A., K. Okabe, P. Polakis, R. Halenbeck, F. McCormick and A. M. Brown, 1990, ras p21 and GAP inhibited coupling of muscarinic receptors of atrial K^+ channels. Cell 61:769-776

Zucker, M.B. and V.T. Nachmias, 1985, Platelet activation. Arteriosclerosis 5: 2-18