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TARGETS OF CYCLIC GMP IN BLOOD PLATELETS: PHOTOLABELLING, MUTAGENESIS AND PHARMACOLOGICAL ANALYSIS OF THE CYCLIC GMP-INHIBITED PHOSPHODIESTERASE

By

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A Thesis

Submitted to the School of Graduate Studies

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ABSTRACT

The first objective of this thesis was to investigate the targets of cyclic GMP (cGMP) action in platelets. Proteins that bind cGMP were first detected by photoaffinity labelling with [^{32}P]cGMP and subsequently identified by molecular, pharmacological and immunological criteria. Since cGMP was already known to exert major effects in platelets through the cGMP-inhibited phosphodiesterase family (PDE3) (Maurice and Haslam, 1990a), an additional objective was to explore the molecular basis of the unique properties of this enzyme by cloning and mutagenesis studies.

A photolabelling technique using [^{32}P]cGMP was modified to permit the rapid detection of cGMP-binding proteins in crude platelet extracts. Five labelled proteins (110, 80, 55, 49 and 38 kDa) were detected in platelet supernatant and four (80, 65, 49 and 38 kDa) in platelet membranes. The sensitivity of photolabelling to PDE3 inhibitors and specific immunoprecipitation established that the 110 kDa photolabelled species was a product of the PDE3 gene family. In addition, the 80 kDa species was identified as cGMP-dependent protein kinase (PKG) by similar methods. Interestingly, cyclic AMP (cAMP) greatly enhanced the labelling of the 80 kDa protein, suggesting the existence of a novel co-operative interaction between cAMP and cGMP. This study also detected a previously unknown 65 kDa cGMP-binding protein in platelet membranes. Since both cAMP and cGMP inhibited labelling of this protein, it may

represent a novel target for both cyclic nucleotides in platelets, possibly a subunit of a cyclic nucleotide-gated (CNG) ion channel.

The inhibitory effects of various compounds on the photolabelling of PDE3 were quantitated by [^{32}P]cGMP. Thus, concentration-dependent inhibition of photolabelling of PDE3 was observed with trequinsin (IC_{50} = 13 $\pm$ 2 nM), lixazinone (IC_{50} = 22 $\pm$ 4 nM), milrinone (IC_{50} = 56 $\pm$ 12 nM), cilostamide (IC_{50} = 70 $\pm$ 9 nM), siguazodan (IC_{50} = 117 $\pm$ 29 nM) and 3-isobutyl 1-methylxanthine (IBMX) (IC_{50} = 3950 $\pm$ 22 nM). The effects of these phosphodiesterase inhibitors on iloprost-stimulated cAMP accumulation in intact platelets were also investigated. Discrepancies between the abilities of these compounds to inhibit photolabelling of PDE3 and to increase platelet cAMP accumulation are probably related to differences in the rates of entry of the individual inhibitors into the intact platelet. The general applicability of this photolabelling technique and its value in the detection of novel cGMP-binding proteins in crude cell extracts was demonstrated in rat tissues. Distinctive photolabelling patterns were observed in different rat tissues and the 110 kDa protein found in human platelets was replaced by a 115 kDa species in rat platelets.

To clarify the molecular mechanisms by which cGMP and inhibitory drugs modulate PDE3 activity, an attempt was made to define the roles of different PDE3 domains in the action of the enzyme. To that end, the C-terminal half of platelet PDE3 was cloned, identified as a product of the PDE3A gene and expressed as an

active enzyme in *E.coli*. Further deletion mutants were generated by removing either an additional 100 amino acids from the N-terminus or the 44 amino acid insert, characteristic of members of the PDE3 family, from the catalytic domain. Site-directed mutagenesis of the 44 amino acid insert was also conducted to explore the function of this region. Kinetic analysis of these mutant enzymes demonstrated that the deletion of N-terminal sequences from PDE3 was accompanied by progressively lower K_m values and by an increased V_{max} for cGMP relative to that for cAMP. Thus, N-terminal sequences exert modulatory effects on cGMP hydrolysis. Deletion of the 44 amino acid insert abolished enzyme activity, as did site-directed mutagenesis of putative β-turns located at the N- and C-termini of the insert. Mutation of a cluster of negatively charged residues in the insert did not have major effects on the hydrolysis of cAMP or cGMP. The results suggest that this insert is required to preserve an effective catalytic domain structure in PDE3.

In conclusion, the major targets of cGMP in platelets were shown to include not only PKG, but also PDE3A and an unidentified membrane protein, possibly a CNG ion channel. This thesis has also identified some of the functionally and structurally important domains within PDE3A.

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DEDICATION

This thesis is dedicated to my parents Lawson and Mary Tang Kong, and to my darling Lloyd Hutchinson for their steadfast support and encouragement throughout all of my endeavours.

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LIST OF ABBREVIATIONS

AA	arachidonic acid
AC	adenylyl cyclase
ADP	adenosine 5′-diphosphate
PKA	cAMP-dependent protein kinase
ATP	adenosine 5′-triphosphate
$[Ca^{2+}]_i$	intracellular calcium concentration
CaM	calmodulin
cAMP	adenosine cyclic 3′,5′-monophosphate
cGMP	guanosine cyclic 3′,5′-monophosphate
DAG	*sn*-1,2-diacylglycerol
DDA	2′,5′-dideoxyadenosine
GC	guanylyl cyclase
PKG	cGMP-dependent protein kinase
G-protein	guanine nucleotide binding protein
GDP	guanine 5′-diphosphate
GP	glycoprotein
GTP	guanine 5′-triphosphate
HEL	human erythroleukemia
IP_3	inositol 1,4,5-trisphosphate

MLC	myosin light chain
MLCK	myosin light chain kinase
NO	nitric oxide
NOS	nitric oxide synthase
PCR	polymerase chain reaction
PDE	phosphodiesterase
PDE III	older nomenclature for PDE3
PGD_2	prostaglandin D_2
PGE_1	prostaglandin E_1
PGI_2	prostacyclin
PIP_2	phosphatidylinositol 4,5-bisphosphate
PKC	protein kinase C
PLA_2	phospholipase A_2
PLC	phospholipase C
ODQ	1*H*-[1,2,4]oxadiazolo[4,3,-*a*]quinoxalin-1-one
SIN-1	3-morpholino-sydnonimine
SNP	sodium nitroprusside
TxA_2	thromboxane A_2
vWF	von Willebrand factor

Acknowledgement of contributions made by others.

Contributions were made by Judith L. Sherwood and Elliot K. Jang as follows:

Paper 1 - photolabelling experiments relating to figures 1 and 2 were conducted by Judith Sherwood.

Paper 2 - measurements of cAMP increases reported in table 1 and figure 7 were conducted by Elliott Jang.

Paper 3 - Elliott Jang provided technical assistance with the enzyme assays.

CHAPTER 1

GENERAL INTRODUCTION

1. Structure and Physiology of Platelets

1.1 Platelet structure

Platelets play an essential role in haemostasis and thrombosis. The interaction between platelets and a damaged blood vessel wall triggers a complex series of processes that minimize blood loss and lead to repair of the site of injury. Resting or inactivated platelets (1 μm x 3 μm) circulate as discoid anuclear cells (reviewed by Blockmans et al., 1995). Extensive invaginations of the plasma membrane serve to increase the surface area of the platelet and produce a surface-connected open canalicular system (White, 1987). In addition to receptors for a wide range of platelet agonists [e.g. thrombin, thromboxane A_2 (TxA_2), and ADP], a variety of membrane glycoproteins (GPs), that function as integrin and non-integrin receptors involved in platelet aggregation and adhesion, have also been identified in the platelet phospholipid bilayer. These glycoproteins include GPIb, a von Willebrand factor (vWF) receptor, GPIa/IIa ($\alpha_2\beta_1$ integrin) and GPVI which are collagen receptors, GPIc/IIa ($\alpha_5\beta_1$ integrin), a fibronectin receptor, and GPIIb/IIIa ($\alpha_{IIb}\beta_3$ integrin), a fibrinogen receptor that also acts as a secondary binding site for vWF (reviewed by Clemetson, 1995).

The platelet membrane skeleton, located directly beneath the plasma membrane, is composed of short actin filaments cross-linked by actin binding proteins (reviewed by Fox, 1993). This skeletal structure is anchored to the membrane by an association with the platelet glycoproteins Ia/IIa and Ib. Both the membrane skeleton and a

circumferential band of microtubules, contribute to maintenance of the discoid shape of the resting platelet (reviewed by Fox, 1993). An extensive intracellular network of membrane-bound vesicles is quite prominent. This dense tubular system regulates the intracellular calcium concentration ($[Ca^{2+}]_i$) by sequestering and releasing Ca^{2+} ions (Siess, 1989). Platelets also contain numerous cytoplasmic organelles including mitochondria, glycogen particles, peroxisomes, lysosomes and two platelet-specific secretory granules. α-Granules contain proteins such as β-thromboglobulin, fibrinogen, fibronectin, platelet-derived growth factor, thrombospondin and vWF, whereas dense granules contain serotonin, ATP, ADP and Ca^{2+} (Zucker and Nachmias, 1985; Siess, 1989).

1.2 Platelet physiology

Circulating platelets do not normally adhere to the endothelial cells lining the vessel wall. However, injury to the vessel wall exposes the underlying connective tissue and allows platelets to interact with collagen and vWF. This adherence of platelets to exposed collagen provides both a physical barrier to blood loss and a site of attachment for additional platelets. Once activated, platelets undergo a series of reactions including shape change, aggregation and secretion (reviewed by Siess, 1991). During shape change, platelets become more spherical and long, spike-like pseudopodial projections are formed. Platelet adherence and shape change are reversible events which are usually followed by one of two forms of aggregation. Primary (reversible) aggregation is generally elicited by a weak stimulus and occurs without a release reaction (secretion of granule constituents), whereas secondary

(irreversible) aggregation requires a stronger stimulus and is associated with TxA_2 synthesis and granule secretion.

In addition to the extensive cytoskeletal reorganization that accompanies the shape change response, a rapid expression of fibrinogen receptors (GPIIb/IIIa) is also detected on the platelet surface (reviewed by Blockmans, 1995). GPIIb/IIIa is a transmembrane molecule consisting of a complex of α- and β- integrin subunits. The α-subunit is composed of an extracellular 125 kDa heavy chain connected to a transmembrane 22 kDa light chain, whereas the β-subunit consists of a single transmembrane protein of 95 kDa (Fitzgerald et al., 1987). Despite the large number of GPIIb/IIIa molecules (40,000-80,000) present on the surface of the resting platelet, binding to fibrinogen only occurs in the activated platelet, since only in the latter is there a conformational change in the GPIIb/IIIa molecule that permits fibrinogen binding. The mechanism responsible for this conformation change is not fully understood. However, changes in intracellular levels of Ca^{2+} and cyclic nucleotides have been implicated in the regulation of GPIIb/IIIa (Shattil and Brass, 1987; Hillery et al., 1991). Fibrinogen, as a bipolar molecule, is capable of binding to GPIIb/IIIa receptors on adjacent platelets, so forming fibrinogen bridges, which are necessary for platelet aggregation. A similar process of interplatelet bridge formation has also been suggested for vWF (Ruggeri, 1993). Under conditions of high shear stress, as is the case in flowing blood, platelet adhesion and aggregation are thought to depend largely on the interaction between platelet GPIb and vWF. Binding of vWF to GPIb has been suggested to open membrane Ca^{2+} channels and increase intracellular Ca^{2+} levels,

which would subsequently induce a conformational change in GPIIb/IIIa, enabling vWF to bind and form molecular bridges (Ruggeri, 1993). Thus, under high stress conditions, both the binding to GPIIb/IIIa and the formation of intercellular bridges are mediated by vWF.

One of the early steps involved in platelet activation is the phosphorylation of myosin light chains (MLC) by the Ca^{2+}/calmodulin dependent protein kinase, MLCK (Daniel et al., 1981; 1984). This phosphorylated form of myosin can now interact with actin, which activates the myosin ATPase activity (Adelstein and Conti, 1975). The energy generated by the breakdown of ATP enables actin and myosin filaments to slide past each other and produce a contractile force. Since myosin and actin accumulate around the granules during the early cytoskeletal restructuring phase, the contractile force generated after myosin phosphorylation serves to transport these organelles towards the platelet centre. Immediately following granule centralization, granule membranes fuse to those of the open canalicular system and the granule contents are secreted (reviewed by White, 1987). Although dense granule contents are readily secreted, α-granule release may require higher agonist concentrations. Thrombospondin, an α-granule constituent, is thought to be involved in the stabilization of the fibrinogen bridges between platelets and is therefore essential for the establishment of irreversible aggregation (Leung and Nachman, 1986). Moreover, other granule constituents, such as ADP, serotonin, fibrinogen and fibronectin play critical roles in the recruitment of platelets to the platelet aggregate (reviewed by Zucker and Nachmias, 1985).

1.3 Signal transduction pathways in platelet activation

Platelet activation can be induced by a variety of stimuli that may be physiological (e.g. collagen, thrombin, ADP) or pharmacological (e.g. vasopressin). These agonists exert their effects by interacting with specific platelet receptors. Most aggregating agents function to increase intracellular Ca^{2+} by triggering Ca^{2+} release from internal stores and/or the opening of membrane channels to allow Ca^{2+} entry (reviewed by Siess, 1989). The intracellular free Ca^{2+} level is critical for the activation status of the platelet. Small increases in Ca^{2+} levels are associated with shape change, whereas higher Ca^{2+} levels result in platelet aggregation and the secretion of granule contents. Many of the receptors involved in platelet signal transduction are coupled by guanine nucleotide-binding proteins (G-proteins) to second messenger generating systems (adenylyl cyclase and phospholipase C). These systems, in turn, trigger physiological responses through phosphorylation events and changes in enzymatic activities.

The agonist receptors that are coupled to G-proteins consist of a single polypeptide chain with an N-terminal extracellular domain, seven hydrophobic transmembrane domains and an intracellular C-terminal domain. The role of heterotrimeric G-proteins, consisting of α-, β- and γ-subunits, in platelet signal transduction has been well-established (reviewed by Brass et al., 1993). The α-subunit, containing the GTP-binding site and GTPase activity, was initially thought to represent the predominant mediator for receptor-effector coupling. However, recent studies have identified important roles for the $\beta\gamma$-complex in the stimulation of

phospholipase C (PLC) activity (Camps et al., 1992) and in the regulation of adenylyl cyclases (Sunahara et al., 1996). To date, 16 subtypes of G_α, 5 subtypes of G_β and 9 subtypes of G_γ have been identified (Brass et al., 1993). A wide range of G-proteins have been detected in platelets including G_i species, G_s, G_q and G_z. The binding of an agonist to the membrane receptor promotes the exchange of GDP for GTP within the heterotrimeric G-protein complex. Upon GTP-binding to the α-subunit in exchange for GDP, the βγ-heterodimer dissociates, thus making both G_α and the $G_{\beta\gamma}$ complex available to interact with specific effector systems. Termination of this interaction results from the intrinsic GTPase activity of the α-subunit, which then reassociates with the βγ-subunit (Spiegel, 1987). More recently, RGS (regulator of G-protein signalling) proteins have been implicated in the deactivation of G-protein mediated signal transduction (Watson et al., 1996). Binding of RGS proteins to selective G_α-subunits has been shown to stimulate GTP hydrolysis to give the GDP-bound form, which in turn reassociates with the βγ-subunit to terminate the signal.

Thrombin is the most potent platelet agonist available, causing shape change, aggregation and secretion from α-granules, dense granules and lysosomes. A thrombin receptor has been cloned and shown to consist of an N-terminal extracellular domain, seven transmembrane segments and a cytoplasmic domain (Vu et al., 1991). The distinguishing feature, of this otherwise typical G-protein-coupled receptor, was the ability of thrombin to cleave the N-terminus of the receptor, thus generating a tethered ligand for receptor activation. Interestingly, platelets from a thrombin-receptor knockout mouse retained the ability to undergo thrombin-stimulated aggregation and

secretion suggesting the presence of a second receptor (Connolly et al., 1996). Thrombin-induced activation of platelet PLC has been well-documented and demonstrated to be GTP-dependent (Hrbolich et al., 1987; Culty et al., 1988) and the specific G-proteins that are involved include G_q and G_i (reviewed by Brass et al., 1993). In platelets, PLC catalyses the breakdown of phosphatidylinositol 4,5-bisphosphate (PIP_2) into diacylglycerol (DAG) and inositol 1,4,5-trisphosphate (IP_3). Newly formed IP_3 molecules bind to an IP_3-receptor to mobilize Ca^{2+} from the dense tubular system. Indirectly, discharge of Ca^{2+} from intracellular stores opens plasma membrane channels to promote an influx of extracellular Ca^{2+} (Irvine, 1990; Berridge, 1993). DAG, also produced from the action of PLC, is an essential physiological regulator of protein kinase C (PKC) activity. Specific isoforms of PKC are translocated from the cytoplasm to the membrane and are activated by DAG (Wang et al., 1995). In the presence of Ca^{2+} ions and phosphatidylserine, PKC will transfer a phosphate from ATP to the serine/threonine residue of specific downstream proteins (Nishizuka, 1984). The principle substrate of PKC in platelets is pleckstrin, a 40 kDa protein of uncertain function.

An early study conducted by Yamanishi et al. (1983) demonstrated that phorbol esters (TPA) and Ca^{2+}-ionophores (A23187) could selectively and independently induce PKC activity and Ca^{2+} mobilization, respectively. These investigators also showed that the exogenous addition of a synthetic DAG to intact platelets, directly activated PKC without inducing Ca^{2+} mobilization (Kaibuchi et al., 1983). In these studies, Nishizuka and colleagues demonstrated that PKC activation and Ca^{2+}

mobilization interact synergistically to mediate platelet activation. More recently, Walker and Watson (1993) utilized a selective inhibitor of PKC (Ro 31-8220) to demonstrate that the synergy between Ca^{2+} and PKC determines the extent of platelet secretion.

The activation of PLA_2 and secretion of ADP from dense granules form critical components of the amplification mechanism for platelet activation. Stimulation of platelets with thrombin leads to the phosphorylation and activation of PLA_2, which is a downstream target of the p38 MAP kinase (Kramer et al., 1995). Once activated, PLA_2 cleaves phospholipids (phosphatidylcholine, phosphatidylethanolamine) at the 2-acyl position to release arachidonic acid (AA) and lysophospholipid (McKean et al., 1981). AA is then quickly converted to prostaglandin cyclic endoperoxides and subsequently to TxA_2 by the sequential actions of cyclo-oxygenase and thromboxane synthase. TxA_2, released by the activated platelet, interacts with a 37 kDa G-protein-coupled receptor protein to cause shape change, aggregation and secretion (Hirata et al., 1991). The G-protein, G_q, links this receptor back to the PLC system, thus further contributing to the amplification mechanism. Recently, two isoforms of the TxA_2 receptors (TxR_α and TxR_β) were identified in human platelets (Hirata et al., 1996). Despite a difference in the size of the carboxyl terminal tails (15 a.a. for TxR_α; 79 a.a. for TxR_β), both receptors display similar ligand binding and PLC activation capabilities. However, distinctive regulatory effects on adenylyl cyclase (AC) activity were demonstrated by these two isoforms. Interaction with the TxR_α receptor isoform activated the AC enzyme, whereas binding to the TxR_β receptor isoform inhibited

activity.

The GPIIb/IIIa integrin is a receptor for adhesive proteins, such as fibrinogen, vWF, vitronectin and fibronectin. Upon ligand binding, the integrin transduces a signal to the inside of the cell (outside-in signaling), by an as yet, undefined mechanism. It is clear, however, that the cytoplasmic domains of the integrin subunits are involved in this process. Phosphorylation of receptor subunits is a well-documented mechanism by which cell surface receptors transmit signals across the membrane. Indeed, the cytoplasmic domain of the β_3-subunit of GPIIb/IIIa contains serine, threonine and tyrosine residues which represent potential phosphorylation sites for protein kinases. Studies conducted by Law et al. (1996) demonstrate that the β_3-subunit is tyrosine-phosphorylated in response to thrombin-induced platelet aggregation. Moreover, the signalling proteins that associate with synthetic peptides corresponding to the phosphorylated cytoplasmic β_3 domain, were identified as GRB2 and Shc, suggesting that tyrosine phosphorylation of β_3 recruits phosphotyrosine binding proteins to the membrane for intracellular signal transduction. A similar process of signal transduction involving tyrosine phosphorylation has also been described in T-cell, B-cell and Fc receptor complexes (Weiss and Littman, 1994). An immune receptor-tyrosine based activation motif (ITAM), containing two tyrosine residues, was demonstrated to play a critical role in the recruitment of signalling molecules (e.g. $pp72^{syk}$) to immune receptors at the membrane surface (Rowley et al., 1995). Although none of the known platelet agonist or adhesion receptors contain tyrosine activation motifs (TAMs), rapid activation of $pp72^{syk}$ was apparent upon

platelet stimulation. The mechanism of $pp72^{syk}$ phosphorylation is unclear, but clustering of platelet Fc receptors with ligand or anti-Fc antibodies has been shown to cause receptor tyrosine phosphorylation and $pp72^{syk}$ association (reviewed by Jackson et al., 1996). In addition to $pp72^{syk}$, platelets also contain numerous other non-receptor tyrosine kinases including src, FAK (focal adhesion kinase) and JAK (Janus kinase).

1.4 Signal transduction pathways for inhibition of platelet function

1.4.1 cAMP

Increases in intracellular cAMP levels have been known for some time to inhibit platelet aggregation (Haslam et al., 1978; Siess, 1989). cAMP is synthesized from ATP through the actions of adenylyl cyclases (AC) (see Section 2.1). Two regulatory G-protein families, G_s and G_i, are responsible for stimulating and inhibiting AC activity and hence cAMP formation. The heterotrimeric G_s protein is coupled to receptors for prostaglandins that inhibit platelet function (PGI_2, PGE_1, PGD_2) and to receptors for adenosine. The latter binds to specific receptors on the platelet surface with both high (K_D=0.16 μM) and low affinity (K_D=2.9 μM) (Huttemann et al., 1984). Once bound, adenosine stimulates AC via the regulatory G_s protein to increase cAMP content by up to 10-fold. The prostaglandins PGI_2, PGE_1 and PGD_2 are similarly coupled to AC via the heterotrimeric G_s protein. Studies have shown that PGI_2 and PGE_1 interact with the same platelet receptor (IP receptor), whereas PGD_2 associates with a distinct platelet receptor (DP receptor) (reviewed by Blockmans, 1995).

Numerous studies have established that cAMP fulfills Sutherland's criteria as the second messenger mediating the inhibition of platelet aggregation by PGE_1 (Marquis et al., 1969; Wolfe and Shulman, 1969; Ball et al., 1970). The supporting evidence included the following findings: 1) PGE_1 activates AC, 2) cAMP increases occur prior to the inhibition of aggregation, 3) inhibitors of cAMP phosphodiesterases can enhance both cAMP accumulation and inhibition of platelet aggregation, and 4) cAMP analogues [e.g. N^6,2′-O-dibutyryl cAMP] can inhibit platelet aggregation. The important contribution made by cAMP to this signalling pathway was confirmed in studies utilizing the adenylyl cyclase inhibitor 2′,5′-dideoxyadenosine (DDA) (reviewed by Haslam et al., 1978). Thus, the inhibition of vasopressin or ADP-induced platelet aggregation by PGE_1 was effectively blocked by the addition of DDA (Haslam et al., 1977; 1978). Moreover, the associated increases in cAMP were also markedly reduced in the presence of this inhibitor, Thus prostaglandins, such PGE_1 act via AC to increase cAMP accumulation and inhibit platelet aggregation.

Since increases in cAMP were associated with inhibition of platelet aggregation, it was initially proposed that decreases in cAMP might promote platelet aggregation (Salzman and Levine, 1971). Support for this possibility came from studies suggesting that some aggregating agents decrease cAMP (Salzman, 1972). Indeed, epinephrine and ADP were demonstrated to interact with the G_i regulatory protein to inhibit AC and the increases in cAMP caused by PGE_1 (Marquis et al., 1970; Cole et al., 1971). However, studies utilizing the AC inhibitor DDA, indicated that inhibition of AC activity did not induce platelet aggregation (Haslam et al., 1978).

Moreover, even when DDA was combined with low concentrations of aggregating agents (collagen, ADP and vasopressin), no potentiation of the platelet aggregation response was observed. Thus, although increases in cAMP concentrations are associated with inhibition of platelet aggregation, decreases in cAMP below resting levels do not promote platelet activation.

1.4.2 cGMP

Although the biologic role of cGMP in platelets is now well-documented, early studies led to much controversy. Thus, agonists such as thrombin and collagen increased cGMP levels in platelets suggesting that cGMP could play a role in platelet aggregation (Jakobs et al., 1974; Chiang et al., 1976; Davies et al., 1976). However, subsequent studies by Haslam et al. (1978; 1980) dissociated platelet aggregation and the secretion of platelet granule constituents from cGMP accumulation, and established that increases in cGMP were an effect rather than a cause of platelet aggregation.

In platelets, nitric oxide (NO) stimulates soluble guanylyl cyclase (GC) to synthesize cGMP from GTP (Drewett and Garbers, 1994). This free radical gas, derived from L-arginine, has effects on platelet aggregation, blood vessel tone, immune function and neurotransmission (see Section 2.2). Since the isoform of NO synthase (eNOS) that catalyses NO synthesis in the endothelium can be stimulated by Ca^{2+}/calmodulin (Bredt and Snyder, 1990), the reported increases in cGMP during platelet activation could likewise be attributed to Ca^{2+} mobilization.

A variety of compounds have been identified which can activate GC by releasing NO. Nitroglycerin, sodium nitroprusside (SNP) and 3-morpholino-synonimine (SIN-1) all generate NO, which diffuses across the cell membrane to interact with GC (Walter et al., 1989). Several studies have shown that the inhibitory effects of NO, SNP and molsidomine on platelet aggregation are closely related to GC activation and cGMP accumulation (Haslam et al., 1980; Mellion et al., 1981; Nishikawa et al., 1982). Moreover, Kadowitz and co-workers demonstrated that S-nitrosothiol compounds dose-dependently inhibited platelet aggregation, activated GC and elevated intracellular cGMP concentrations (Mellion et al., 1983). These effects were partially reversed in the presence of methemoglobin, a hemoprotein that binds NO. Collagen-induced platelet aggregation has been shown to be accompanied by increases in cGMP (Radomski et al., 1990). Addition of the NO synthase inhibitor N^G-monomethyl-L-arginine blocked the increase in cGMP and potentiated aggregation. In contrast, addition of L-arginine, the substrate for NO synthesis, enhances cGMP accumulation and inhibited aggregation. Thus, stimulation of platelets induced NO synthesis and cGMP accumulation. Taken together, these studies suggest that platelet activation triggers a negative feedback mechanism, in the form of increased cGMP, that may regulate platelet reactivity. More recently, the identification of a potent and selective inhibitor of soluble GC (ODQ : 1*H*-[1,2,4]oxadiazolo[4,3,-*a*]quinoxalin-1-one) has enabled these investigators to confirm that, directly or indirectly, cGMP mediates the antiaggregatory actions of NO (Moro et al., 1996). Treatment of collagen stimulated platelets with ODQ effectively inhibited cGMP accumulation induced by NO and restored platelet aggregation. Thus, ODQ blocks the actions of NO by

inhibiting soluble GC and decreasing cGMP.

The role for cGMP in the inhibition of platelet adhesion remains poorly understood. Early studies by Radomski et al. (1987) demonstrated that platelet adhesion to collagen fibers and the vascular endothelium was completely blocked by NO. More recently, NO was shown to reduce platelet adhesion to the endothelium under flow conditions (de Graff et al., 1992). Exposure of platelets to SNP has been demonstrated to increase both cGMP (9-fold) and cAMP (3-fold) under rapid flow conditions (Polanowska-Grabowska and Gear, 1994). Since the 3-fold increase in cAMP accounted for less that 20% of the observed inhibition of adhesion, the increase in cGMP must be primarily responsible for the reduced adhesion.

2. Regulation of cyclic nucleotide synthesis

2.1 cAMP

Since cAMP is formed from ATP by the actions of AC, regulation of AC activity is an important factor in determining the final intracellular concentration of cAMP. It is well-established now that three distinct classes of proteins (membrane receptors, G-proteins and AC) interact to define cAMP synthesis. At present, ten ACs have been cloned and the deduced amino acid sequences predict a complex structure which spans the membrane twelve times (Krupinski et al., 1989; Sunahara et al., 1996). Two large cytosolic domains (loop 3 and the C-terminal tail) containing putative ATP-binding sites are homologous between the different types of ACs (50-92 %), whereas little or no homology exists between the transmembrane domains

(Sunahara et al., 1996). Moreover, a cooperative interaction between these two ATP-binding sites has been suggested by studies which monitored the effects of expression of the two ATP-binding domains simultaneously and separately (Tang et al., 1991). A Ca^{2+}/calmodulin binding site, adjacent to the plasma membrane, is predicted to exist in the first cytoplasmic loop of the Ca^{2+}-stimulated type I AC (Vorherr et al., 1993).

Although it is clear that G-proteins (G_s and G_i) regulate the activity of ACs, the mechanisms by which this is accomplished are not well-defined. The $G_{s\alpha}$-subunit-GTP complex, generated when the agonist binds to the receptor, interacts with and activates all isoforms of AC (reviewed by Jackson, 1991). The extent to which AC remains active is determined by the rate of GTP hydrolysis by $G_{s\alpha}$, thus making the GTPase activity of the G-protein the rate determining step in AC activation (Levitzki, 1987). In contrast, when the heterotrimeric G_i protein is activated by agonist-receptor interactions, inhibition of AC activity may result. The activated $G_{i\alpha}$-subunit proteins (α_{i1}, α_{i2}, α_{i3}) can effectively inhibit type V or type VI AC activity, whereas the $G_{\beta\gamma}$-subunit complex is a potent inhibitor of the type I AC (reviewed by Sunahara et al., 1996). Interestingly, $G_{\beta\gamma}$ has also been shown to stimulate the activity of selected isoforms of AC (type II and type IV).

All receptors that interact with G-proteins consist of a single subunit with seven internal hydrophobic domains that form transmembrane α-helices (Dohlman et al., 1991). These transmembrane domains assemble to form a hydrophobic pocket containing a ligand-binding site. Most of the early work defining the receptors

coupled to AC was focused on adrenergic receptors (Dohlman et al., 1991). The β-adrenergic and α_2-adrenergic receptors are coupled to AC through G_s and G_i respectively, whereas the α_1-adrenergic receptors are coupled via G_q to the PLC system. In platelets, agonists such as thrombin and epinephrine, interact with seven-transmembrane-segment receptors and G_i to inhibit AC activity. As discussed previously, adenosine and the prostaglandins, PGI_2 and PGE_1, interact with A_2 and IP receptors, respectively, to elevate cAMP levels (Leigh et al., 1984; Barrington et al., 1989).

2.2 cGMP

The synthesis of cGMP from GTP is catalysed by guanylyl cyclases (GC). Two types of GC have been defined, particulate forms and soluble forms (reviewed by Drewett and Garbers, 1994). Most cells contain both types of GC; however, only soluble GC activity has been detected in platelets (Adams and Haslam, 1978). The particulate GCs consists of an N-terminal extracellular ligand-binding domain, a single transmembrane domain, a protein kinase-like ATP-binding domain and a single catalytic domain. To date, six isoforms of membrane GCs (GC-A through GC-F) have been cloned (reviewed by Garbers, 1994; Fulle et al., 1995) and ligands for the first three forms have been defined. Atrial and brain natriuretic peptides (ANP/BNP), members of the type A and B natriuretic peptide families, preferentially bind to the GC-A isoform, whereas members of the type C natriuretic peptide family associate with the GC-B isoform. Both GC-A and GC-B are distributed widely throughout the body (reviewed by Drewett and Garbers, 1994). In contrast, the receptor for

guanylin/heat-stable enterotoxin, GC-C, is predominantly found in the intestinal epithelium (Vaandrager and De Jonge, 1994). The GC-D isoform is localized to olfactory tissues (Fulle et al., 1995), whereas the GC-E and GC-F isoforms appear to be retina-specific (Margulis et al., 1993). Although ligands for these recent three isoforms have yet to be identified, enzyme activity is very sensitive to intracellular Ca^{2+} concentrations. At low Ca^{2+} levels, guanylyl cyclase-activating proteins (GCAPs), stimulate GC activity.

The structure and function of the particulate GCs (especially GC-A) have been extensively studied over the last decade (reviewed by Drewett and Garbers, 1994). As expected, the extracellular ligand binding domains are highly divergent between the different GC isoforms, whereas the cytoplasmic regions appear to be more closely related. Two functional domains are present in the cytoplasmic tail of GC, a protein kinase-like domain and a cyclase (or catalytic) domain that is highly homologous to both of the cyclase domains present in AC (Krupinski et al., 1989). Although protein kinase activity has not been associated with the GC receptors, deletion of the protein kinase domain generates a constitutively active enzyme that is no longer regulated by ANP (Chinkers and Garbers, 1989). Thus, these investigators suggest that the protein kinase domain functions as a negative regulatory element.

Cytosolic GC is a heterodimer (α/β) with subunit molecular masses of 82 kDa (α) and 70 kDa (β) (Kamisaki et al., 1986). Additional isoforms of the α- and β-subunits have been identified by molecular cloning (Yuen et al., 1990; Harteneck et

al., 1991; Giuili et al., 1992). At present, six different subunits (α_1 - α_3 and β_1 - β_3) have been cloned. However, only the α_1/β_1 heterodimer has been isolated from tissue extracts (reviewed by Garbers et al., 1994). *In vitro* expression of α- and β-subunits, both of which contain catalytic domains, indicated that GC activity required heterodimer formation (Harteneck et al., 1990; Buechler et al., 1991). Moreover, the coexpression of α_2- and β_1-subunits generated a catalytically active heterodimer (Harteneck et al., 1991).

Activators of soluble GC include free radicals such as nitric oxide (NO), hydroxyl radicals and carbon monoxide (CO), as well as oxidized fatty acids and porphyrins (Ignarro, 1989; Drewett and Garbers, 1994). An enzyme known as nitric oxide synthase (NOS) catalyses the conversion of L-arginine to nitric oxide (Currie et al., 1983). Three classes of NOS have been identified, a constitutively expressed neuronal form (nNOS), a constitutively expressed endothelial form (eNOS) and a cytokine inducible form (iNOS) (Knowles and Moncada, 1994). Although very little is known about the molecular basis of GC activation by NO, it is clear that a prosthetic heme group functions as a receptor for NO binding (Gerzer et al., 1981; Humbert et al., 1990) and CO binding (Verma et al., 1993). Interaction of NO with the heme moiety of GC is postulated to displace the iron from the plane of the porphyrin ring, resulting in a conformational change in the protein that activates the enzyme. Recently, studies conducted by Wedel and co-workers (1994) demonstrated that mutation of His-105 in the β_1-subunit, yielded a NO-insensitive form of GC. Since His-105 is located upstream of the putative catalytic domain, these investigators

suggest that binding of heme, and hence NO, is likely to occur in this region. Since platelets contain only the soluble form of GC, compounds that release NO can alter the intracellular levels of cGMP and influence the activation status of the platelet. As will be discussed in Section 4, cGMP can regulate cell function by effects on the activities of protein kinases, cyclic nucleotide phosphodiesterases and ion channels.

3. Regulation of cyclic nucleotide degradation

Phosphodiesterases (PDEs) catalyse the hydrolysis of cyclic 3′,5′-nucleotides to inactive 5′-monophosphates by breaking a phosphodiester bond of the cyclic phosphate ring. Regulation of the steady-state levels of cAMP and cGMP within cells by PDEs, allow these enzymes to regulate the biological effects of these cyclic nucleotides. In many tissues, cyclic nucleotide degradation usually requires the coordinated efforts of several distinct but structurally related PDE isoenzymes. As a result of the identification of increasing numbers of new PDE isotypes, a universal and systematic method for their classification was required.

3.1 Classification and nomenclature of PDEs

PDEs were originally classified according to their elution profiles on a DEAE-cellulose anion exchange column (Thompson and Appleman, 1971; Hidaka and Asano, 1976; Weishaar et al., 1986; Beavo, 1988). Since the individual peaks obtained using these chromatographic procedures often contained more than one PDE isoenzyme, as well as catalytically-active proteolytic fragments, the resultant enzymatic analyses and characterizations were inaccurate and confusing. Recently, a standardized system of

nomenclature was adopted, which incorporated the relevant structural information from the individual enzymes (Beavo, 1995). Each PDE is now given an eight-character designation (e.g. HSPDE1A1), in which the first two letters represent the species, the next three letters and arabic numeral identify the PDE gene family and the remaining letter and arabic numeral indicated the specific gene product and splice variant. Using this classification, seven distinct mammalian PDE gene families have so far been defined (PDE1 to PDE7; Table 1).

3.2 PDE1 gene family (Ca^{2+}/CaM-PDE)

The PDE1 family currently consists of three structurally related genes (PDE1A, PDE1B and PDE1C), each of which has been shown to possess alternate splice variants. The bovine heart (59 kDa) (Ho et al., 1977; Hansen and Beavo, 1982) and brain (61 kDa) (Sharma et al., 1984) enzymes, for instance, have identical amino acid sequences except at the N-terminus, indicating that they were derived from the same gene (PDE1A) by tissue specific alternate splicing (Charbonneau et al., 1991; Novack et al., 1991; Sonnenburg et al., 1993). In contrast, the 63 kDa bovine brain enzyme (Sharma et al., 1984) was found to be distinct from the 61 kDa isoenzymes and hence was defined as PDE1B (Bentley et al., 1992). Structurally-related family variants of PDE1B have also been detected in brain, kidney and adrenal medulla (Bentley et al., 1992; Repaske et al., 1992; Sonnenburg et al., 1993). Both the PDE1A and PDE 1B isoforms exhibit a higher affinity for cGMP than for cAMP as substrate (Wang et al., 1990; Wu et al., 1992). The PDE1C isoform was the most recent subfamily member to be detected. At least five 3′-spice variants of this gene have already been identified

Table 1 Characteristics of the Phosphodiesterase Gene Families

Gene Family	Substrate	Regulatory Properties	Inhibitor Sensitivities
PDE1	cGMP > cAMP	Calcium, Calmodulin	Vinpocetine, 8-Methoxymethyl-IBMX
* PDE2	cAMP $\simeq$ cGMP	stimulated by cGMP	EHNA
* PDE3	cAMP >> cGMP	inhibited by cGMP	lixazinone milrinone cilostamide
PDE4	cAMP	phosphorylated by PKA	rolipram
* PDE5	cGMP >> cAMP	phosphorylated by PKG	zaprinast
PDE6	cGMP	activated by transducin	none known
PDE7	cAMP	-	none known

* present in platelets

(Loughney et al., 1994; Yan and Beavo, 1994). Moreover, the 75 kDa variant expressed in the rat olfactory neuroepithelium demonstrated a high affinity for cAMP, suggesting that activation of this enzyme would have a major impact on intracellular cAMP levels and possibly play an important role in olfactory signal transduction (Yan and Beavo, 1994).

The kinetic properties of the PDE1 enzymes reported in early studies differed significantly from laboratory to laboratory, probably due to the varying degrees of enzyme purity (Lin and Cheung, 1980). Although some studies were carried out using purified preparations of enzyme, it was later determined that contaminating PDE1 variants were present (Beavo, 1995), thus making the K_m and V_{max} determinations suspect. Since even a small amount of contamination by an isoenzyme with a high affinity for one cyclic nucleotide could greatly alter the relative ratios of cAMP and cGMP hydrolysis (Beavo, 1995), caution must be exercised when determining kinetic profiles of PDE isozymes in tissues expressing more than one isoform.

All PDE1 gene family members are homodimers requiring Ca^{2+} and calmodulin (CaM) for enzymatic activity. Three distinct domains have been postulated in these gene products, a C-terminal catalytic domain was mapped by analysis of proteolytic cleavage products (Charbonneau et al., 1991), an N-terminal CaM-binding domain was predicted based on the identification of a basic amphipathic helix (Charbonneau et al., 1991; Novack et al., 1991), and an inhibitory domain adjacent to a second CaM-binding domain was recently identified by deletion mutagenesis (Beavo, 1995).

Although it is the removal of the inhibitory action of this domain that results in PDE1 activation, the mechanism by which the Ca^{2+}-CaM complex accomplishes this is not fully understood. It is clear, however, that activation by Ca^{2+}-CaM markedly increases the V_{max} of the enzyme.

A role for phosphorylation in PDE1 regulation *in vivo*, has been suggested given that PDE1 enzymes can be readily phosphorylated *in vitro* (Sharma and Wang, 1985; 1986). Phosphorylation experiments conducted on the purified bovine brain and heart isoenzymes (Sharma and Wang, 1986; Sharma, 1991), indicated that 1), in the presence of the Ca^{2+}-CaM complex, no phosphorylation of PDE1 was detected, 2) a several-fold decreased sensitivity to enzyme activation by the Ca^{2+}-CaM complex coincided with PDE1 phosphorylation, and 3) sensitivity of the enzyme to Ca^{2+}-CaM activation was increased upon dephosphorylation of the enzyme with calcineurin, a Ca^{2+}-CaM-dependent phosphatase. Although the cAMP-dependent protein kinase phosphorylated both Ser-120 and Ser-138 of PDE1, site-directed mutagenesis indicated that phosphorylation of Ser-120 was associated with a reduction in the affinity of the enzyme for the Ca^{2+}-CaM complex (Florio et al., 1994).

3.3 PDE2 gene family (cGS-PDE)

PDE2 was first described in the cytosolic fraction of rat liver (Beavo et al., 1970), but has since been identified in various other tissue types (reviewed by Manganiello et al., 1990b). This enzyme has been purified to near homogeneity from bovine adrenal and cardiac tissues (Martins et al., 1982), bovine and rabbit brain

tissues (Whalin et al., 1988; Murashima et al., 1990), rat liver (Pyne et al., 1987) and human platelets (Grant et al., 1990). PDE2 exists as a homodimer with a subunit molecular mass of 100-105 kDa (Martins et al., 1982; Yamamoto et al., 1983; Trong et al., 1990).

Although PDE2 can hydrolyze both cAMP and cGMP at similar maximal rates, cAMP is the preferred substrate at physiological concentrations of cyclic nucleotides (Martins et al., 1982; Manganiello et al., 1990a). This enzyme exhibits positive homotropic co-operativity and requires Mg^{2+} or Mn^{2+} for optimal catalytic activity. Low concentrations of cGMP (1-5 μM) have been shown to stimulate cAMP hydrolysis by binding to an allosteric site, whereas higher concentrations of cGMP have an inhibitory effect on hydrolysis of cAMP, probably due to competition for the catalytic site (Beavo, 1988; Manganiello et al., 1990a). The binding of cGMP to PDE2 alters the cAMP reaction kinetics by reducing the K_m for cAMP without changing the V_{max} of the enzyme.

Two isoenzymes of PDE2 have been cloned, the first from a bovine adrenal cortex library (Sonnenburg et al., 1991) and the second from a rat brain library (Yang et al., 1994). Sequence analysis of these two enzymes indicated that they are splice variants of the same gene (PDE2A). The brain PDE2 isoenzyme is particulate and differs structurally from the cytosolic adrenal PDE2 enzyme by the presence of an N-terminal hydrophobic sequence that could play a role in membrane targeting. Although phosphorylation experiments with the rabbit brain enzyme (105 kDa)

indicate that it is a substrate for PKA *in vitro*, this post-translational modification had no effect on the catalytic activity of the enzyme (Whalin et al., 1988). Recently, a potent and selective inhibitor of PDE2 was identified by Podzuweit et al. (1995). This compound, *erythro*-9-(2-hydroxy-3-nonyl)adenine (EHNA), originally designed as an inhibitor of adenosine deaminase, demonstrated the ability to inhibit PDE2 activity with an IC_{50} of about 1 μM.

The catalytic and regulatory domains of bovine cardiac PDE2 have been mapped by photoaffinity labelling with [^{32}P]cGMP (Stroop et al., 1989). Cyanogen bromide fragmentation of photolabelled PDE2 identified two cGMP-binding sites, a high-affinity site present in a 28 kDa fragment and a low-affinity site located on a C-terminal 36 kDa fragment. Since addition of cAMP prior to photolabelling selectively inhibited labelling of the 36 kDa cleavage product, and cAMP hydrolytic activity was associated with the photolabelled 36 kDa fragment, the catalytic domain of PDE2 must reside in this region. Comparisons between PDE2 and the rod and cone PDE enzymes, which are members of the PDE6 gene family, revealed a conserved region of 340 residues that contained two internally homologous repeats (Charbonneau et al., 1990). All three of these PDEs share the ability to bind cGMP at a high affinity, non-catalytic site, suggesting a role for cGMP binding in the regulation of PDE activity. Studies conducted by Erneux and co-workers indicate that binding of cGMP to the high affinity regulatory site of PDE2 correlated with the ability of cGMP to potentiate PDE2 enzyme activity (Erneux et al., 1981; Miot et al., 1985). Moreover, cleavage of this allosteric binding site from the PDE2 enzyme generated a catalytically active

fragment that was no longer stimulated by cGMP (Stroop et al., 1989). Thus, PDE2 can be described as a chimeric molecule containing a regulatory domain that binds cGMP and a catalytic domain that is activated by binding of cGMP to the regulatory domain.

3.4 PDE3 gene family (cGI-PDE)

The PDE3 enzyme is present in a variety of tissues including platelets, heart, liver, vascular smooth muscle and adipose tissue. The subcellular distribution of PDE3 is cytosolic in platelets (Grant and Colman, 1984; MacPhee et al., 1986) and predominantly membrane-associated in the remaining tissue types (Degerman et al., 1987; Pyne et al., 1987; Meacci et al., 1992). PDE3 has been detected as a homodimer in platelets (Grant and Colman, 1984), myocardium (Harrison et al., 1986; Beavo, 1988) and liver (Pyne et al., 1987; Boyes and Loten, 1988). Western blot analysis and immunoprecipitation studies have identified the PDE3 holoenzyme as having a subunit molecular mass of 110 kDa in platelets (MacPhee et al., 1986; Grant et al., 1988), 138 kDa in placenta (LeBon et al., 1992) and 130-135 kDa in cardiac and adipose tissues (Degerman et al., 1990; Smith et al., 1991; Smith et al., 1993).

Members of the PDE3 gene family possess relatively high affinities for both cAMP and cGMP (K_m values: 0.2 - 0.4 μM and 0.02 - 0.8 μM, respectively) (Manganiello et al., 1990a). However, the roughly 10-fold lower V_{max} for cGMP makes PDE3 much more effective at cAMP hydrolysis than at cGMP hydrolysis (Grant and Colman, 1984; MacPhee et al., 1986; Degerman et al., 1990). As a result,

the cAMP hydrolytic activity of PDE3 can be competitively inhibited by cGMP and the PDE3 enzymes have been commonly referred to as cGMP-inhibited phosphodiesterases (cGI-PDEs). In addition to cGMP, there exists a large number of compounds with the ability to inhibit PDE3 activity selectively. The expression of cGI-PDE in platelets, heart and vascular smooth muscle has stimulated the development of PDE3 inhibitors capable of blocking platelet aggregation, increasing cardiac contractility and relaxing vascular smooth muscle (Harrison et al., 1986; MacPhee et al., 1986). These compounds have potential cardiotonic, anti-thrombotic and anti-hypertensive activities. Published IC_{50} values for widely used PDE3 inhibitors studied over the last decade range from 10-53 nM for lixazinone (Venuti et al., 1988; Chapter 4, this thesis) and 40-80 nM for cilostamide (MacPhee et al., 1986; Degerman et al., 1990) to 460-860 nM for milrinone (MacPhee et al., 1986; Venuti et al., 1988; Degerman et al., 1990).

Since PDEs usually exist as minor proteins in cells, their purification including that of PDE3, has usually involved large quantities of starting materials, as well as sequential chromatographic steps (Grant and Colman, 1984; MacPhee et al., 1986). Human platelet PDE3, for example, was first purified by Grant and Colman (1984). Soluble platelet proteins were released by homogenization and collected after high speed centrifugation. Purification was accomplished in two steps. First, the supernatant was applied to a DEAE cellulose column and eluted with a sodium acetate gradient (0 - 1M). Second, column fractions containing PDE activity were identified, pooled and applied to a Blue-dextran-Sepharose column; elution using a cAMP

gradient (0 - 1 mM) yielded purified PDE3 (Grant and Colman, 1984).

More recently, many investigators have capitalized on the currently available molecular cloning techniques to assist in the characterization of PDE3. Two distinct genes (PDE3A and PDE3B) have been recently identified within this family (Meacci et al., 1992; Taira et al., 1993). PDE3A cDNA sequence was obtained using oligonucleotide probes based on partial platelet amino acid sequences to screen a human cardiac cDNA library (Meacci et al., 1992). A clone containing an open reading frame of 3423 bps was eventually isolated and predicted a protein with a molecular mass of 125 kDa. Sequence analysis of this clone identified six putative membrane-association segments at the N-terminus and a C-terminal catalytic domain, containing a 44 amino acid insertion not found in other PDE gene families. PDE3B was identified by screening a rat adipocyte cDNA library (Taira et al., 1993). The deduced nucleotide sequence of the 123 kDa rat adipocyte clone was sufficiently different from the previously published human cardiac sequence to rule out an alternate splicing mechanism. The presence of a large hydrophobic domain at the N-terminus of this protein also suggested a possibility of membrane-association.

A 74 kDa placental PDE3 enzyme was recently cloned and found to be identical in sequence to the C-terminus of the human heart enzyme (Kasuya et al., 1995). Baculovirus expression of this placental cDNA yielded 60% of the cGI-PDE activity in the supernatant fraction. Moreover, immunostaining of Sf9 insect cells expressing this enzyme generated a cytosolic staining pattern. Further analysis

suggested that an alternate transcription start site was responsible for the production of this cytosolic form of cGI-PDE. In addition, these investigators found that the absence of the N-terminus significantly increased the K_m value of the enzyme. However, kinetic analysis of N-terminal truncation mutants of cardiac PDE3 expressed in Sf9 cells suggested no significant change in K_m relative to the intact enzyme (Leroy et al., 1996).

The 110 kDa platelet PDE3 enzyme is also cytosolic and the isolation of a clone expressing the entire sequence for the soluble enzyme is actively being pursued. Since completion of the work on which this thesis is based, one group (Cheung et al., 1996) has reported the isolation of cDNA clones expressing PDE3A sequences from a human erythroleukemia (HEL) cell cDNA library. The longest clone represented 87% of the cardiac PDE3A cDNA and was 100% identical at the nucleotide level. Sequences corresponding to the remainder of the cardiac enzyme were amplified by reverse transcriptase PCR (RT-PCR). Using total platelet RNA and RT-PCR, these investigators also demonstrated that corresponding cDNA sequences, as well as sequences representing the N-terminus of the cardiac enzyme (1-536) could be amplified from platelet material. Thus, both platelets and HEL cells contain PDE3 mRNA sequences coding for a full length (125 kDa) cardiac enzyme. It remains to be determined whether or not the 110 kDa platelet PDE3A protein represents a truncated form of the 125 kDa cardiac enzyme.

Shortly after their discovery, PDE3 enzymes were shown to be regulated by hormones and growth factors (Loten and Sneyd, 1970; Loten et al., 1978). Hormonal stimulation of PDE3 isoenzymes in hepatocytes (Marchmont et al., 1981), adipocytes (Degerman et al., 1990) and platelets (MacPhee et al., 1988) were associated with enzyme phosphorylation and activation. Moreover, incubation of the PDE3B isoenzyme in rat adipocytes with okadaic acid, a phosphatase inhibitor, resulted in enzyme activation, indicating that a phosphorylation event was involved in enzyme regulation (Shibata et al., 1991). The activation of phosphatidylinositol 3-kinase by insulin has been implicated as an upstream event in the regulation of PDE3 phosphorylation (Rahn et al., 1994). In this study, wortmannin, a selective inhibitor of phosphatidylinositol 3-kinase, was shown to block the activation/phosphorylation of PDE3B in rat adipocytes. Sequence analysis of the recently cloned PDE3B cDNA identified several consensus PKA phosphorylation sites at the N-terminus of the molecule (Taira et al., 1993). Studies conducted on rat adipocytes initially identified Ser-427 residue as the site of phosphorylation *in vitro*. However, more recently, it was determined that Ser-302 and not Ser-427 was phosphorylated in response to insulin and/or isoproterenol in the intact adipocyte (Rahn et al., 1996).

3.5 PDE4 gene family (cAMP-PDE)

The PDE4 gene family represents the largest of the seven currently defined PDE gene families, with more than 30 isoenzymes identified to date. Four distinct genes (PDE4A, PDE4B, PDE4C and PDE4D) have been identified in rats and subsequently in humans (reviewed by Muller, 1996). Members of this rapidly

expanding PDE gene family have a high affinity and selectivity for cAMP and a sensitivity to inhibition by certain antidepressant drugs, such as rolipram and Ro 20-1729 (Conti and Swinnen, 1990). Multiple variants, derived by alternate splicing, have been detected in three subfamilies, with the variants of the PDE4D gene being the best characterized. Four distinct polypeptides were generated from the PDE4D gene, PDE4D1, PDE4D2, PDE4D3 and PDE4D4. The PDE4D1 and PDE4D2 isoforms were identified as 5′-splice variants with estimated molecular masses of 72 kDa and 67 kDa (Monaco et al., 1994). Moreover, alternate splicing in the first intron (84 bp) of the PDE4D gene produced a frame shift that was responsible for changing the position of the ATG initiation site of the PDE4D2 variant. Expression of both PDE4D1 and PDE4D2 isoforms were driven by an intronic promoter present in testicular Sertoli cells (Monaco et al., 1994), whereas expression of the PDE4D3 variant (93 kDa) appeared to be controlled by an upstream promoter found in thyroid and brain tissues (Sette et al., 1994a). The fourth variant (PDE4D4), detected in human and rat, is currently under investigation; the existence of another distinct promoter has been suggested (Conti et al., 1995).

The functional significance of these PDE4D variants was explored by comparing the properties of the 67 kDa, 72 kDa and 93 kDa gene products. Sette and colleagues demonstrated that stimulation of rat thyroid cells with thyroid stimulating hormone led to short term phosphorylation and activation of the constitutively expressed PDE4D3 (93 kDa) enzyme (Sette et al., 1994a) and induced the *de novo* synthesis of the PDE4D1 (72 kDa) and PDE4D2 (67 kDa) variants (Sette et al.,

1994b). Thus, regulation of the 72 kDa and 67 kDa proteins occurred at the level of mRNA transcription, whereas activation of the 93 kDa isoform involved a phosphorylation mechanism. It is possible that PDE phosphorylation provided the rapid decrease in cAMP levels required for a short-term response, with the long-term feedback regulation being achieved by modification of mRNA transcription. It remains to be determined whether this versatility in PDE regulation also exists for the other PDE4 family members.

Since the differences between the three isoforms of PDE4D occurred at the N-terminus, it seemed likely that the sensitivity of the 93 kDa protein to cAMP-dependent phosphorylation resulted from the presence of the additional 132 amino acids. Recently, site-directed mutagenesis of the PDE4D3 variant identified Ser-54 as the phosphorylation site involved in enzyme activation (Sette and Conti, 1996). Moreover, phosphorylation increased the maximal velocity of cAMP hydrolysis (V_{max}) without altering the K_m for cAMP. A change in the pattern of inhibition by rolipram was also detected in the phosphorylated enzyme. Measurement of unphosphorylated PDE4D activity in the presence of increasing concentrations of rolipram, generated a biphasic curve demonstrating high affinity inhibition at 10^{-9} M rolipram and low affinity inhibition at 10^{-6} M rolipram (Sette and Conti, 1996). Phosphorylation of PDE4D enhanced the enzyme activity that is inhibited by rolipram with a high affinity. The relationship between the high affinity and low affinity rolipram binding sites is still not clear. In one model, two binding sites for rolipram were postulated, a high affinity binding site and a cAMP-hydrolytic site (Muller et al., 1996). In an alternate

model, two conformational states for PDE4 were suggested, a high affinity form and a low affinity form (Muller et al., 1996; Sette and Conti, 1996).

3.6 PDE5 gene family (cGB-PDE)

The PDE5 enzyme was first detected and characterized in lung extracts (Lincoln et al., 1976; Francis et al., 1980) and has since been identified in a variety of tissue types including platelets (Hamet et al., 1984), rat spleen and vascular smooth muscle (reviewed by Francis et al., 1990). This enzyme has been purified from rat and bovine platelets (Coquil et al., 1980; Robichon, 1991) and lung extracts (Thomas et al., 1990). Both lung and platelet PDE5 enzymes are homodimers with reported subunit molecular masses of 93 and 95 kDa, respectively. The lung PDE5 enzyme hydrolyses cGMP with a K_m of 5 μM (Thomas et al., 1990), whereas the K_m for cGMP reported for the platelet enzyme was much lower (0.22 μM, Robichon, 1991).

The similarity between the PDE5 and PDE6 enzymes with respect to size, cGMP-binding properties and kinetic characteristics, suggested that there could also be sequence similarities between these two families. Surprisingly, a comparison of the cloned bovine lung PDE5 sequence with the photoreceptor PDE6 sequence indicated less than 60% identity (McAllister-Lucas et al., 1993). However, a high affinity non-catalytic cGMP-binding site, containing two internally homologous repeats, was found N-terminal to the catalytic domain in both of these PDE gene families. The relationship between this upstream cGMP-binding site and catalytic domain remains unclear. However, binding of cGMP to this site shifted the elution of the protein to a

later position in the gradient, suggesting that a conformational change in the enzyme had occurred (Thomas et al., 1990). Moreover, when this high affinity site is occupied by cGMP, the PDE5 enzyme is rapidly phosphorylated on Ser-92 by PKG (Thomas et al., 1990). Thus, the conformational change in the N-terminal domain, resulting from cGMP binding, probably provided the protein kinase with access to otherwise hidden phosphorylation sites within the enzyme. The functional significance of this phosphorylation event is currently under investigation. Although one group has reported that treatment of partially purified preparations of guinea pig lung enzyme with PKA appeared to enhance cGMP hydrolytic activity (Burns and Pyne, 1992a; Burns et al., 1992b), this stimulatory effect could not be reproduced using purified PDE5 preparations, suggesting that an additional cellular factor was required for the observed activation.

More recently, a role for Zn^{2+} in the regulation of PDE5 activity has been suggested by Corbin and colleagues. These investigators demonstrated that Zn^{2+} bound with high affinity to PDE5 and was necessary for catalytic activity (Francis et al., 1994). A sequence motif ($Hx_3Hx_{20}E$), present in zinc hydrolases, was identified in PDE5 and postulated to function as a Zn^{2+} binding site. In addition, the observation that the PDE5 inhibitor, zaprinast, was a chelator of Zn^{2+}, suggested that the selective inhibitory action of this compound could be partly due to an ability to remove Zn^{2+} (Pyne, 1994).

3.7 PDE6 gene family (rod/cone-PDE)

Gene products of the PDE6 family are highly expressed in the outer segments of the retinal photoreceptor neurons. To date, three subfamilies (PDE6A, PDE6B and PDE6C) have been cloned and identified. The rod membrane-associated PDE is the most abundant of the photoreceptor isoenzymes and exists as 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, that interact to form the catalytic region ($PDE_{\alpha\beta}$). Association of $PDE_{\alpha\beta}$-subunits with two inhibitory γ-subunits (11 kDa) inactivates the enzyme. Most of the rod PDE6 enzyme is membrane-associated, though a soluble form has also been detected in bovine photoreceptors (Gillespie and Beavo, 1989). The cone PDE6 enzyme is likewise a cytosolic enzyme. Although the subunit stoichiometry of cone PDE6 is not well-defined, a complex involving two identical α'-subunits (94 kDa), two distinct γ-subunits (11 kDa and 13 kDa) and a novel δ-subunit (15 kDa) has been detected (Gillespie and Beavo, 1989).

The physiological role of PDE6 in the phototransduction process is now very well understood (Stryer, 1991; Lagnado and Baylor, 1992). In the retinal rod, exposure to light modifies rhodopsin so that an interaction with the heterotrimeric GTP-binding protein, transducin ($T_{\alpha\beta\gamma}$), is favoured. This interaction catalyses the exchange of bound GDP for free GTP, thus stimulating the dissociation of the complex ($T_{\alpha\beta\gamma}$-GTP) into T_α-GTP and $T_{\beta\gamma}$-subunits. The T_α-GTP, in turn, activates PDE6 by blocking the inhibitory action of the γ-subunits. The active PDE6 enzyme lowers the intracellular cGMP concentration, and so decreases cGMP binding to the cGMP-gated

cation channels. Since the channels are open when cGMP is bound, lowering the cGMP levels closes these channels and effectively causes hyperpolarization of the cell and an eventual reduction in neurotransmitter release.

Members of the PDE6 family contain a high-affinity, non-catalytic cGMP binding site similar to those present in the PDE2 and PDE5 gene families. A role for this site in the regulation of PDE6 activity has been suggested (Arshavsky et al., 1992; Cote et al., 1994). When cellular concentrations of cGMP are low so that PDE6 cGMP-binding sites are empty, a rapid dissociation of the $PDE_{\alpha\beta\gamma}$-T_α-GTP complex into catalytically active ($PDE_{\alpha\beta}$) and inactive (PDE_γ-T_α-GTP) fragments is seen. Alternatively, when cGMP binding sites are occupied, as is the case with high levels of intracellular cGMP, the T_α-GTP subunit blocks the inhibition by the γ-subunits, but remains bound in a complex with the PDE heterotetramer. Since the GTPase activity of T_α-GTP is lower when it is associated with the PDE6 holoenzyme, the latter remains enzymatically active longer. Thus, the local cGMP concentration can have an impact on the duration of PDE6 activation (Arshavsky et al., 1992). The termination of PDE6 activity was also thought to involve phosphorylation of the γ-subunit (Hayashi et al., 1991; Takemoto et al., 1993; Yamazaki et al., 1996). Phosphorylation of Thr-35 in the bovine γ-subunit by PKC appeared to increase the inhibitory activity of the γ-subunit, possibly by enhancing its affinity for the catalytic subunits (Udovichenko et al., 1994). Similar mechanisms of PDE6 inactivation by γ-subunit phosphorylation reported by others involve a phosphatidylinositol-stimulated kinase (Hayashi et al., 1991), or a frog retinal outer segment kinase (Tsuboi et al., 1994).

3.8 PDE7 gene family

The PDE7 family is the most recent and consequently least well-defined of the seven PDE gene families. cDNA encoding a truncated protein expressing cAMP hydrolytic activity was cloned from a human glioblastoma cDNA library (Michaeli et al., 1993). The deduced amino acid sequence of this enzyme fragment most closely resembled that of the PDE4 family (35% identity, 51% similarity). Although the expressed PDE7 enzyme exhibited a high affinity for cAMP (K_m = 0.2 μM), it was not inhibited by the typical PDE4 inhibitors, such as rolipram, nor by PDE3 inhibitors such as milrinone (Michaeli et al., 1993). Northern blot analysis detected abundant expression of PDE7 mRNA in human skeletal muscle with lower levels observed in brain and cardiac tissues. More recently, several investigators have demonstrated the presence of high affinity cAMP-specific PDE7 activity in T-lymphocytes (Ichimura and Kase, 1993; Bloom and Beavo, 1994).

3.9 Platelet phosphodiesterases

Purification of three kinetically distinct PDEs from platelet cytosol by DEAE-cellulose chromatography was first reported by Hidaka and Asano (1976). Three peaks of enzyme activity were designated as Form I, Form II and Form III, according to their order of elution from the DEAE-cellulose column. It has now been established that these forms represent members of the PDE5, PDE2 and PDE3 families. Although there has been one report that Form II contained two isoenzymes (PDE1 and PDE2) (Simpson et al., 1988), additional support for this view is not

available (Weishaar et al., 1986).

In platelets, isoenzymes from three PDE gene families function in a concerted manner to regulate cyclic nucleotide hydrolysis. PDE5, purified from bovine platelets (Robichon, 1991), exhibited characteristics similar to those of the previously identified lung enzyme (Thomas et al., 1990). Both the platelet and lung isoenzymes have a high affinity cGMP-binding site, a selective sensitivity to inhibition by zaprinast and similar monomeric molecular masses (95 kDa). In addition, phosphorylation of both PDE5 enzymes by either PKA or PKG required the presence of cGMP (Robichon, 1991; Thomas et al., 1990).

PDE2 has been purified to homogeneity from human platelet cytosol (Grant et al., 1990) and appears to be structurally and kinetically similar to the soluble bovine adrenal and cardiac isoforms (Martins et al., 1982). The platelet PDE2 enzyme is cytosolic with an estimated subunit molecular mass of 105 kDa. Catalytic activity required Mg^{2+} or Mn^{2+}, but was insensitive to Ca^{2+} or calmodulin stimulation (Grant et al., 1990). Although the V_{max} values for cAMP and cGMP hydrolysis were similar *in vitro*, cAMP was the preferred substrate *in vivo*. Moreover, low levels of cGMP were shown to stimulate platelet cAMP hydrolysis 3 to 10 fold (Grant et al., 1990).

The PDE3 enzyme has been considered to be the major cAMP PDE in platelets and is responsible for more than 80% of the cAMP hydrolytic activity at submicromolar concentrations of cAMP (MacPhee et al., 1986). Although initial

attempts to purify PDE3 from platelets demonstrated the presence of a 61 kDa species (Grant and Colman, 1984), immunoblotting experiments with the bovine platelet protein detected a 110 kDa protein (MacPhee et al., 1986), suggesting that the former species was actually a proteolytic fragment of the latter. Recently, a rapid single step cilostamide affinity column procedure was developed to purify 'intact' (110 kDa) PDE3 enzyme. Although unproteolyzed forms of PDE3 were obtained, the majority (90%) of the purified preparation consisted of fragments ranging in size from 53 kDa to 79 kDa (Degerman et al., 1994). Attempts to clone PDE3 directly from platelets has met with limited success. Instead, one group has used HEL cells as a source of mRNA and cDNA to obtain PDE3 sequence (Cheung et al., 1996). Recently, these investigators isolated a partial cDNA clone from a HEL cell library and showed that it was identical to the cardiac PDE3A enzyme. Additional studies indicated that both HEL cells and platelets contain mRNA sequences encoding the 125 kDa cardiac enzyme (see Section 3.4). As discussed previously, this does not explain the presence of a soluble 110 kDa PDE3A enzyme in platelets.

Regulation of platelet PDE3 activity has been shown to involve a phosphorylation mechanism. Human platelets treated with forskolin (Grant et al., 1988) or prostaglandins (PGE_1 or PGI_2) (Alvarez et al., 1981; MacPhee et al., 1986) demonstrated increased cAMP hydrolysis. Moreover, this increased activity was abolished when the protein kinase inhibitor, H-8, was present (Grant et al., 1988), suggesting the involvement of a phosphorylation event. Studies conducted by MacPhee and co-workers (1988) provided further evidence for the involvement of

phosphorylation in PDE3 regulation. Direct *in vitro* phosphorylation of platelet extracts with the catalytic subunit of cAMP-dependent protein kinase (PKA) produced a similar increase in PDE activity, as did treatment of intact platelets with prostaglandins, indicating that PDE3 activation was mediated by cAMP-dependent phosphorylation. As discussed previously, the rat adipocyte isoform (PDE3B) was phosphorylated and activated upon stimulation with insulin and/or isoproterenol (Degerman et al., 1990). Since platelets also contain receptors for insulin (Falcon et al., 1988), it was possible that this could provide an alternate method of platelet PDE3 regulation. Indeed, studies conducted by Lopez-Aparicio et al. (1992) demonstrated that serine phosphorylation of PDE3 accompanied the treatment of platelets with insulin and increased cAMP hydrolysis. Moreover, this phosphorylation reaction occurred in the presence of H-7, an inhibitor of PKC, PKA and PKG, suggesting the involvement of an insulin-stimulated protein kinase. Thus, two distinct phosphorylation pathways have been implicated in the regulation of platelet PDE3 activity.

Recently, a role for histidine and cysteine residues in PDE3 activity was suggested (Ghazaleh et al., 1996). Two histidine residues are postulated to be necessary for cGMP inhibition, whereas a cysteine residue and two additional histidine residues were required for enzyme activity. The authors proposed that these residues interact with the bivalent cations (Mg^{2+}) that are necessary for PDE3 catalytic activity.

4. Mechanisms of action of cyclic nucleotides

4.1 Cyclic nucleotide dependent protein kinases

4.1.1 cAMP-dependent protein kinases (PKA)

The cAMP-dependent protein kinase (PKA) is the major intracellular receptor for cAMP. By transferring γ-phosphate from ATP to a Ser or Thr residue in the target protein, PKAs play an essential role in the regulation of fundamental cellular processes. The holoenzymes are tetramers (R_2C_2), consisting of a regulatory subunit dimer (R_2) and two catalytic (C) subunit monomers (reviewed by Scott, 1991). Binding of cAMP to the regulatory subunits (R_2) induces a conformational change, which results in the release of two catalytically active subunits (C). Two types of PKAs have been identified based on their order of elution from a DEAE-cellulose column (Type I and Type II) (reviewed by Francis and Corbin, 1994). Although both types of PKAs have similar catalytic subunits, their regulatory subunits (R_I and R_{II}) differ with respect to molecular size, sequence and phosphorylation state. Unlike the R_I-subunit, the R_{II}-subunit can be readily phosphorylated by the catalytic subunit at Ser-95. Phosphorylation effectively decreases the rate of holoenzyme association by lowering the affinity of the R_{II}-subunit for the catalytic subunit (reviewed by Scott, 1991). Four R-subunits isoforms ($R_{I\alpha}$, $R_{I\beta}$, $R_{II\alpha}$ and $R_{II\beta}$) have been identified in mammalian tissues and represent distinct gene products (reviewed by Francis and Corbin, 1994). The R-subunits are composed of functionally distinct subdomains involved in dimerization, inhibition of the catalytic subunit, subcellular localization and binding of cAMP. Located in the C-terminal two-thirds of each R-subunit, are two in-

tandem cAMP binding sites, site A and site B. The N-terminal site A dissociates cAMP relatively rapidly, whereas site B dissociates cAMP much more slowly. Both sites A and B exhibit positive cooperativity to produce synergistic activation of PKA (reviewed by Francis and Corbin, 1994). When a single molecule of cAMP binds to either site A or B, the R-subunit undergoes conformational changes that "prime" the holoenzyme for activation. Once additional cAMP molecules have bound to the remaining sites, further conformation changes are initiated, that ultimately result in the release of active catalytic subunits.

Unlike the dimeric regulatory subunits, the catalytic subunit is a monomeric enzyme. At present, three mammalian C-subunit isoforms have been identified, C_α, C_β and C_γ (reviewed by Beebe, 1994). Moreover, two alternative splice variants of the C_β gene have been detected. Both the C_α and C_β isoforms have a wide tissue distribution, whereas, the C_γ isoform is restricted to the testis and sperm (reviewed by Beebe, 1994). Sequence analysis of the C_α and C_β isoforms indicates a high degree of homology (93% amino acid identity). In contrast, C_γ significantly differs from C_α and C_β, demonstrating only 83% and 79% amino acid identity, respectively (Beebe et al., 1990). The kinetic properties and substrate specificities of the C_α and C_β isoforms are quite similar, whereas those of the C_γ isoform appear quite distinct. Studies conducted by Beebe et al. (1992) indicate that C_γ exhibits a reduced sensitivity to the heat-stable protein kinase inhibitor (PKI), and a preference for histone over the peptide, kemptide, as a substrate. The significance of the unique substrate and inhibitor specificity of C_γ remains to be determined. The catalytic subunit is composed of a large and small lobe

(Knighton et al., 1991). The small lobe, located at the N-terminus consists of antiparallel β-sheets and an ATP-binding site. The large lobe, consisting of an α-helical domain and a small β-sheet, binds the substrate and positions the ATP for transfer of the γ-phosphate.

In many tissues, a significant proportion of PKAs, especially the $Type_{II}$ isoform is membrane-associated (reviewed by Francis and Corbin, 1994). The proteins that interact with the R_{II} subunit to anchor the holoenzyme to the membrane are known as A Kinase Anchoring Proteins (AKAPs). Although AKAPs can vary in size, amino acid sequence and relative affinities for the R_I and R_{II} subunits, the acidic amphipathic helix motif necessary for interaction with the basic residues of the R_{II} subuint remains conserved in all AKAPs. In some cases, AKAPs are themselves substrates for PKA, suggesting that the phosphorylation event may initiate additional AKAP functions involved in cAMP-mediated responses.

The subcellular distribution of PKAs in platelets differs from that in most other cells, in that it is the R_I subunits that are membrane bound and the R_{II} subunits that are cytosolic (Salama and Haslam, 1981; 1984). DEAE cellulose chromatography of soluble platelet proteins produced a single peak of cAMP-dependent activity characteristic of the Type II enzyme. In contrast, extraction with Triton-X 100 was required to isolate the R_I subunit from the particulate platelet fraction, indicating that the Type I enzyme is clearly membrane-bound.

4.1.2 cGMP-dependent protein kinases (PKG)

The cGMP-dependent protein kinase (PKG) is selectively activated by cGMP to transfer γ-phosphate from ATP to Ser or Thr residues of target substrates (reviewed by Hofmann et al., 1992). This enzyme has been shown to mediate relaxation of vascular smooth muscle, reduce cardiomyocyte contractility and inhibit platelet aggregation (reviewed by Walter, 1989). Two types of PKGs have been identified; a soluble form (Type I) and a membrane-bound form (Type II). The Type I enzyme, which is found at high concentrations in platelets, smooth muscle cells and brain tissues, exists as a homodimer consisting of 75 kDa subunits (reviewed by Scott, 1991). Two isoforms of the soluble PKG (Type I_α and Type I_β) have been identified and are derived from the same gene by alternative splicing. Type II PKG, originally isolated from rat intestine, also exists as a dimer, and is thought to be involved in the regulation of ion transport and fluid secretion within the small intestine (Markert et al., 1995).

In contrast to PKA, both the catalytic and regulatory domains of PKG are located in the same subunit structure. The catalytic core, located at the C-terminus, contains the ATP-binding site and the substrate-binding site, whereas the N-terminal regulatory region is composed of a dimerization domain, an inhibitory domain and two cGMP-binding domains (reviewed by Hofmann, 1992). The cGMP-binding sites in PKG are structurally similar to the cAMP-binding sites of PKA. The selectivity of PKG for cGMP is due to the presence of a Thr residue rather than an Ala residue at the cGMP binding site. The hydroxyl group of the Thr residue is postulated to form a hydrogen bond with the 2-amino group of guanine in cGMP. Indeed, studies in which

the Ala residue of PKA was replaced by the Thr residue, resulted in a mutant with an increased affinity for cGMP (Shabb et al., 1990). Although both cyclic nucleotide binding sites of PKG selectively bind cGMP over cAMP, the affinity of one site (site I) for cGMP is higher than the affinity of the second site (site II). In addition, cGMP binding to these sites occurs with positive cooperativity and synergistically activates PKG (reviewed by Vaandrager and De Jonge, 1996). Enzyme activation of PKG proceeds in two stages (reviewed by Scott, 1991). First, cGMP binds to the site adjacent to the catalytic domain (site I) to make PKG 50% active and to "prime" site II for cGMP binding. Subsequent binding of cGMP to site II displaces the inhibitory domain leading to full activation of PKG.

Although PKG is specifically activated by cGMP, cross-activation of PKG by cAMP has been demonstrated in certain tissues. It is well-established that both cAMP and cGMP can induce smooth muscle relaxation (reviewed by Hofmann et al., 1992). However, studies with pig tracheal smooth muscle have demonstrated that kinase-specific analogues of cGMP but not of cAMP induce muscle relaxation, suggesting that in this tissue, it is PKG that mediates the effects of cAMP (Francis et al., 1988). Since autophosphorylation of PKG increases its affinity for cAMP 6-10 fold, and vascular smooth muscle cells contain 5 times more cAMP than cGMP, it seems likely that increases in cAMP could cross-activate PKG (Landgraf et al., 1986). The converse process, in which cGMP cross-activates PKA has also been demonstrated. Thus, elevations in cGMP caused by the heat-stable enterotoxin, increases chloride conductance across the membranes of T-84 colon carcinoma cells (Forte et al., 1992).

Moreover, cAMP analogues that potently activate PKA, also stimulates chloride secretion.

4.1.3 PKA and PKG substrates in platelets

A large variety of proteins are phosphorylated when intact human platelets are exposed to agents that increase cAMP or cGMP. Treatment of ^{32}P-labelled platelets with PGE_1, is associated with the phosphorylation of 22, 24, 36, 49, 50, 130 and 250 kDa proteins (Haslam et al., 1979; 1980). Moreover, the activation of PKG in platelets has also been associated with the phosphorylation of 22, 24, 49 and 50 kDa proteins (Haslam et al., 1980).

The 22 kDa protein phosphorylated in response to increased intracellular levels of cAMP has been identified as rap 1b, a low molecular weight GTP binding protein. Prenylation of the carboxyl-terminal cysteine residue, targets rap 1b to the cell membrane. *In vitro* studies of purified rap 1b demonstrates that phosphorylation by PKA and PKG occurs at Ser 179 (Kawata et al., 1989; Miura et al., 1992). Moreover, treatment of the intact platelets with agents that increase cAMP leads to phosphorylation of rap 1b and the appearance of the phosphoprotein in the cytosol (Siess et al., 1990). Phosphorylation of rap 1b in human platelets is induced by nitric oxide generating compounds (Haslam et al., 1980; Grunberg et al., 1995). The treatment of intact platelets with a nitric oxide donor (S-nitroso-albumin) led to phosphorylation of rap 1b and a dose-dependent inhibition of platelet aggregation. In addition, the combination of SIN-1 and iloprost (agents which increase cGMP and

cAMP, respectively), was shown to phosphorylate rap 1b in human platelets synergistically (Grunberg et al., 1995). Although the function of rap 1b has not as yet been identified, the very slow time course of protein phosphorylation indicates that it is not involved in the inhibition of platelet aggregation induced by collagen, phorbol ester, vasopressin, ADP or epinephrine (Siess and Grunberg, 1993).

The 24 kDa protein was identified as the β-subunit of GPIb, and its phosphorylation has been correlated with inhibition of collagen-induced actin polymerization (Fox, 1993). Indeed, when GPIb is missing from platelets, as is the case in Bernard-Soulier platelets, the PKA-induced inhibition of actin polymerization was not detected.

The 49 and 50 kDa [32P]-labelled species (Haslam et al., 1980) representing different states of phosphorylation of the same protein, is a major substrate of PKA and PKG both *in vitro* and in intact human platelets (Hallbrugge et al., 1990). Phosphorylation of this protein, which shifts its apparent molecular mass from 46 kDa to 50 kDa, is closely correlated with the inhibition of platelet activation and aggregation (Hallbrugge and Walter, 1989). This 46-50 kDa protein, representing a novel microfilament associated protein, has been termed the vasodilator-stimulated phosphoprotein (VASP) (Reinhard et al., 1992). Both PKA and PKG phosphorylate VASP at the same sites (Ser 157, Ser 329 and Thr 278), but, the order of phosphorylation is different for each kinase (Butt et al., 1994; Haffner et al., 1995). PKG preferentially phosphorylates Ser 239, whereas PKA will phosphorylate Ser 157

before Ser 239 (Butt et al., 1994). In addition, it is the phosphorylation of Ser 157 by PKA or PKG, that is responsible for the shift in mobility of VASP to 50 kDa (Butt et al., 1994). Moreover, studies conducted by Horstrup et al. (1994) demonstrates that phosphorylation of VASP at Ser 157 is correlated with the inhibition of fibrinogen binding to GPIIb/IIIa. At present, there is no evidence for a direct interaction between VASP and GPIIb/IIIa.

Recently, VASP was cloned from human HL-60 and canine MDCK cells (Haffner et al., 1995). Three distinct regions composed of two globular N- and C-terminal domains separated by a central proline-rich domain were identified. The central region contains three Ser/Thr phosphorylation sites and four copies of a proline rich motif (GPPPPP). The proline-rich regions of VASP have been suggested to interact with the actin-binding protein known as profilin (Reinhard et al., 1995). Since both profilin and VASP bind directly to actin and are localized to highly dynamic membrane regions, a role in the regulation of microfilament organization has been proposed for VASP (Reinhard et al., 1995). Although a wide variety of signalling proteins are known to bind to proline-rich proteins via SH3 domains, the proline domains of VASP do not demonstrate the typical consensus sequence for SH3 binding (Haffner et al., 1995). A mixed charge cluster, located at the C-terminus of VASP, is predicted to form an α-helical structure. Indeed, deletion of the C-terminus appeared to alter the subcellular distribution of VASP, implying that this region is involved in anchoring VASP at focal adhesion sites (Haffner et al., 1995).

Since Ca^{2+} ions are important mediators of platelet aggregation, modulation of $[Ca^{2+}]_i$ levels by PKA and PKG can have a major impact on platelet function. Changes in $[Ca^{2+}]_i$ levels can be accomplished either by modifying Ca^{2+} transport across the plasma membrane or the dense tubular system. Many agonists including thrombin, TxA_2 and vasopressin, mobilize intracellular stores of Ca^{2+}, as well as stimulate the influx of extracellular Ca^{2+}. Movement of Ca^{2+} inward across the plasma membrane is accomplished by receptor-operated Ca^{2+} channels or store-operated Ca^{2+} channels, and outward Ca^{2+} movement by the plasma membrane Ca^{2+}-ATPase pump. In addition, the IP_3-regulated channel releases Ca^{2+} from the dense tubules, whereas a Ca^{2+}-ATPase pump functions to sequester Ca^{2+}. It is the balance between the activities of these Ca^{2+} channels and pumps that determines the $[Ca^{2+}]_i$. The modes of Ca^{2+} entry are not clearly understood, but it is generally accepted that depletion of intracellular Ca^{2+} stores generates a substantial influx of extracellular Ca^{2+} (Berridge et al., 1995). This phenomenon known as "store-regulated Ca^{2+} influx" was established in studies utilizing the endomembrane Ca^{2+}-ATPase inhibitor, thapsigargin. Treatment of platelets with thapsigargin demonstrated that emptying of the Ca^{2+} stores was accompanied by extracellular Ca^{2+} influx, in the absence of cell surface receptor activation (Sargeant et al., 1992; Heemskerk et al., 1993). The biochemical mechanism for this event is not clear, but there is speculation about the involvement of protein tyrosine kinases, or the formation in the dense tubules of a diffusible messenger (reviewed by Heemskerk and Sage, 1994). In addition, some agonists, such as ADP, may act directly through receptor-operated Ca^{2+} channels (Sage et al., 1990).

The action of cAMP and cGMP on Ca^{2+} movements is actively being explored. Several studies have reported that cyclic nucleotides selectively inhibit thrombin/thromboxane-induced Ca^{2+} entry in platelets, without affecting Ca^{2+} influx secondary to store depletion (Brune et al., 1994; Doni et al., 1994). Moreover, it has also been suggested that increases in cAMP or cGMP stimulates Ca^{2+} extrusion by increasing the activity of the plasma membrane Ca^{2+}-ATPase pump (Tohmatsu et al., 1989; Johansson and Haynes, 1992). Studies conducted by Nakamura et al. (1995) examining the effect of SNP and iloprost on Ca^{2+} entry and release in human platelets, demonstrated that cAMP and cGMP slowed the release of Ca^{2+} from the dense tubules and attenuated external Ca^{2+} entry. Although the mechanism of this effect was not defined, these authors postulate roles for PKA and PKG. Recently, Cavallini et al. (1996) demonstrated that PGI_2 and SNP could inhibit the ability of IP_3 to release Ca^{2+} from intracellular stores. Moreover, this effect was correlated with phosphorylation of the IP_3 receptor, suggesting that cyclic nucleotides prevent the release of Ca^{2+} from the dense tubular system by inhibiting the activity of the IP_3 receptor. Thus, the regulation by cyclic nucleotides of Ca^{2+} release and uptake in platelets is quite complex and remains to be fully defined.

4.2 Cyclic nucleotide-gated (CNG) ion channels

Cyclic nucleotide-gated (CNG) ion channels were first identified ten years ago when a cation channel in the retinal rod photoreceptor was shown to be directly activated by cGMP (reviewed by Finn et al., 1996). Prior to that discovery, the effects of cyclic nucleotides on ion channels were generally believed to result from channel

phosphorylation by cyclic nucleotide-dependent protein kinases (PKA and PKG). CNG ion channels are now known to have a widespread distribution and have been detected not only in visual and olfactory receptor neurons, but also in aorta, heart, brain, kidney and colon (reviewed by Finn et al., 1996). These channels are activated by the binding of a ligand (cAMP or cGMP) and hence functionally resemble ligand-gated channels. However, structurally, CNG channels demonstrate greater similarity to the voltage-gated channels.

CNG channels are hetero-oligomers composed of at least two different subunits (α and β). The α-subunit consists of six membrane spanning domains, cytoplasmic N- and C-terminal regions and a pore forming loop located between membrane segments 5 and 6 (reviewed by Zimmerman, 1995). In addition, a stretch of 80-100 amino acids, located in the C-terminus of the channel, is structurally homologous to the cyclic nucleotide binding domains of PKA and PKG. The cyclic nucleotide-binding sites of the hetero-oligomeric channel complex are assembled into a pocket containing an α-helix and multiple β-strands (reviewed by Zimmerman, 1995). cAMP or cGMP is stabilized in this pocket by hydrogen bonds and non-polar interactions with the channel protein. The visual CNG channels are 30 - 40 times more sensitive to cGMP than to cAMP, whereas the olfactory channels display similar sensitivities to both cyclic nucleotides (reviewed by Distler et al., 1994).

The β-subunit, which is homologous to, and co-purifies with the 63 kDa α-subunit, has been identified as a 240 kDa protein, capable of binding Ca^{2+}/calmodulin

(Korschen et al., 1995). The cDNA representing the entire β-subunit of the rod CNG channel has been recently cloned (Korschen et al., 1995). Sequence analysis of the 240 kDa polypeptide indicates an unusual bipartite structure, consisting of an amino region that is identical to a glutamic acid rich protein (GARP), and a carboxyl region that is highly homologous to the α-subunit. The function of the GARP domain has not as yet been defined, although potential roles in protein-protein interactions or rod membrane targeting have been postulated (Korschen et al., 1995). Although expression of the α-subunits alone can generate functional CNG channels, co-expression of α- and β-subunits produces a channel which more closely resembles the native CNG channels. It is not clear how many α- and β-subunits are required to form the channel. However, sequence homology to the voltage-gated channels predicts that the CNG channels exists as tetramers. The activation of a rod CNG channel requires that at least three molecules of cGMP bind to the complex. Sequence analysis of both α- and β-subunits demonstrates the existence of putative cGMP binding sites at the C-terminus. Moreover, photoaffinity labelling with the cGMP analog, 8-(*p*-azidophenacylthio)-[^{32}P]cGMP, specifically labelled both the 63 kDa α-subunit and the 240 kDa β-subunit (Brown et al., 1993; 1995). Thus, both subunits bind cGMP and are likely to contribute to CNG channel activation.

The movement of Ca^{2+} through CNG channels is physiologically important in both the visual and olfactory signal transduction systems. The $[Ca^{2+}]_i$ concentration within the rod photoreceptor is determined by the balance between Ca^{2+} entry through the open CNG channels and Ca^{2+} extrusion by the Na^+/Ca^{2+}-K^+ exchanger. The

absorption of light by the visual pigment rhodopsin triggers a series of events which ultimately results in a decreased intracellular cGMP concentration, closure of the CNG channel and hyperpolarization of the cell. In contrast, signal transduction within the cilia of the olfactory receptor neuron begins with closed CNG channels, which open upon stimulation with odorants (reviewed by Zufall et al., 1994). The odorant molecule activates a G-protein-mediated signal transduction cascade that stimulates AC to produce cAMP, which in turn, opens the CNG channels permitting Ca^{2+} entry. This Ca^{2+} current then activates a depolarizing Cl^- current, thus amplifying the initial response.

CNG channels were originally thought to be limited to cells and tissues involved in sensory signal transduction. However, the currently available northern blotting and PCR techniques have led to the detection of rod CNG α-subunit sequences in heart, kidney and colon, and olfactory CNG sequences in aorta and heart tissues (reviewed by Finn et al., 1996). Recently, the α-subunit of the cone CNG channel was cloned from bovine kidney (Biel et al., 1994). Sequence analysis of the cone α-subunit revealed 60% and 62% amino acid identity to the rod and olfactory CNG channel proteins, respectively. PCR and northern analysis indicated that cone CNG α-subunit sequences were present in testis, kidney and heart. The cloned cone sequence was transiently expressed in human embryonic kidney 293 cells and the electrophysiological properties of the resultant CNG ion channels were examined. cGMP was shown to activate selectively the CNG ion channels, which in turn permitted the inward movement of Ca^{2+} ions. Thus, the presence of CNG channel

protein sequences in nonsensory cells suggests that some of the functional effects of cGMP in these cells could be mediated by activation of CNG ion channels.

Recently, sequences corresponding closely to the rod photoreceptor CNG α-subunit were detected in human megakaryocytes (Surks et al., 1995). A combination of RACE-PCR and plaque screening of a cDNA library was undertaken to obtain a clone with 90% homology to the rod CNG sequence. Electron microscopy and immunogold labelling studies localized this protein to the plasma membrane of platelets and megakaryocytes. The physiological significance of this protein in platelet function remains to be determined. As the identification of CNG sequences in non-neuronal tissues increases, some investigators have suggested that the major function of CNG channels in these tissues is to increase $[Ca^{2+}]_i$ concentrations, rather than to regulate the membrane potential.

4.3 Cyclic nucleotide phosphodiesterases

4.3.1 PDE2

Early indications that regulation of PDE2 by cGMP was physiologically important came from studies on frog ventricular cells (Hartzell and Fischmeister, 1986; Fischmeister and Hartzell, 1990) and bovine glomerulosa cells (MacFarland et al., 1991). Investigation of primary cultures of adrenal glomerulosa cells suggested a role for PDE2 as a mediator of ANP-induced decreases in cAMP and steroidogenesis. By using a patch clamp technique, Fischmeister and co-workers also demonstrated that the enhanced Ca^{2+} current resulting from increases in intracellular cAMP levels could be

effectively reduced by introducing cGMP. Since a specific inhibitor of PDE2 was unavailable at that time, these workers could only speculate that PDE2 was the target of cGMP in these cells. More recently, the identification of EHNA as a selective PDE2 inhibitor has enabled these investigators to demonstrate that the inhibitory effect of cGMP on the cAMP-stimulated Ca^{2+} current is mediated by PDE2 (Mery et al., 1995). EHNA was shown to reverse the inhibition caused by cGMP in a dose-dependent manner (IC_{50} = 3 μM). In addition, the inhibitory effects of SIN-1 and SNP on the Ca^{2+} current was blocked by 30 μM EHNA.

An investigation of the role of PDE2 in the rat pulmonary circulation was conducted by Haynes et al. (1996). These workers showed that EHNA reversed the pulmonary vasoconstriction in the perfused rat lung caused by hypoxic gas challenge. Since the EHNA-induced vasodilation was not blocked by adenosine receptor antagonists, an alternative mechanism of action by EHNA was proposed. Partially purified PDE2 preparations from rat pulmonary artery were used by these investigators to demonstrate that the dose-dependent inhibition of cGMP-stimulated activity by EHNA was correlated with the dose-dependent EHNA-induced vasodilation in the isolated rat lung. Thus, the ability of EHNA to reverse the hypoxic pressor response in the perfused rat lung appeared to be mediated in part by the activation of PDE2.

Two groups have reported effects of cGMP on endothelial cell permeability, one using porcine pulmonary artery cells (Suttorp et al., 1996) and a second using rat coronary artery cells (Hempel et al., 1996). Endothelial cell permeability, as measured

by movements of albumin, was shown to increase when rat cells were exposed to agents that activate AC (isoproterenol). In contrast, stimulation of GC by ANP, SIN-1 and SNP reduced cellular cAMP and decreased the movement of albumin across the membrane. Similar effects were also reported using porcine pulmonary artery endothelial cells exposed to hydrogen peroxide (Suttorp et al., 1996). Increases in cGMP resulting from pretreatment of these cells with ANP or dibutyryl cGMP blocked the hydrogen peroxide-induced endothelial cell permeability, whereas addition of a nitric oxide synthase inhibitor effectively restored endothelial cell permeability. Moreover, GC activation and PDE2 inhibition were shown to act synergistically to reduce permeability.

In platelets, increases in cAMP levels have been known to inhibit platelet activation (see Section 1.4.1). Intracellular levels of cAMP are determined by the balance of AC and PDE activities. The actions of cyclic nucleotides on platelet PDEs can have a major impact on platelet function. cGMP has been shown to inhibit cAMP breakdown in intact platelets by acting on PDE3 (see Section 4.3.2). Since PDE2 is also present in platelets, it could likewise represent an important target for cyclic nucleotide action. Recent studies from this laboratory have investigated the role of PDE2 in the regulation of cAMP in human platelets (Dickinson et al., 1996). The addition of EHNA to platelets enhanced the increases in cAMP caused by high but not low SNP concentrations, suggesting that PDE2 restricted cAMP accumulation in the presence of cGMP. Interestingly, SNP had an inhibitory effect on cAMP accumulation with high PGI_2 concentrations, suggesting that under these conditions, activation of

PDE2 by cGMP had a greater impact on cAMP accumulation than the inhibition of PDE3 by the former cyclic nucleotide.

4.3.2 PDE3

Synergism between activators of AC and nitrovasodilators was first described by Levin et al. (1982). These authors demonstrated the ability of SNP and PGI_2 to act synergistically to inhibit both platelet aggregation and TxA_2 synthesis. Subsequent studies by Radomski et al. (1987) confirmed the synergistic effects of prostacyclin and NO, and further demonstrated the ability of these compounds to disaggregate collagen-stimulated platelets. More recently, the combination of SIN-1 and the prostacyclin analogue, iloprost, was shown to act synergistically to stimulate phosphorylation of the platelet proteins rap 1b and VASP (Grunberg et al., 1995).

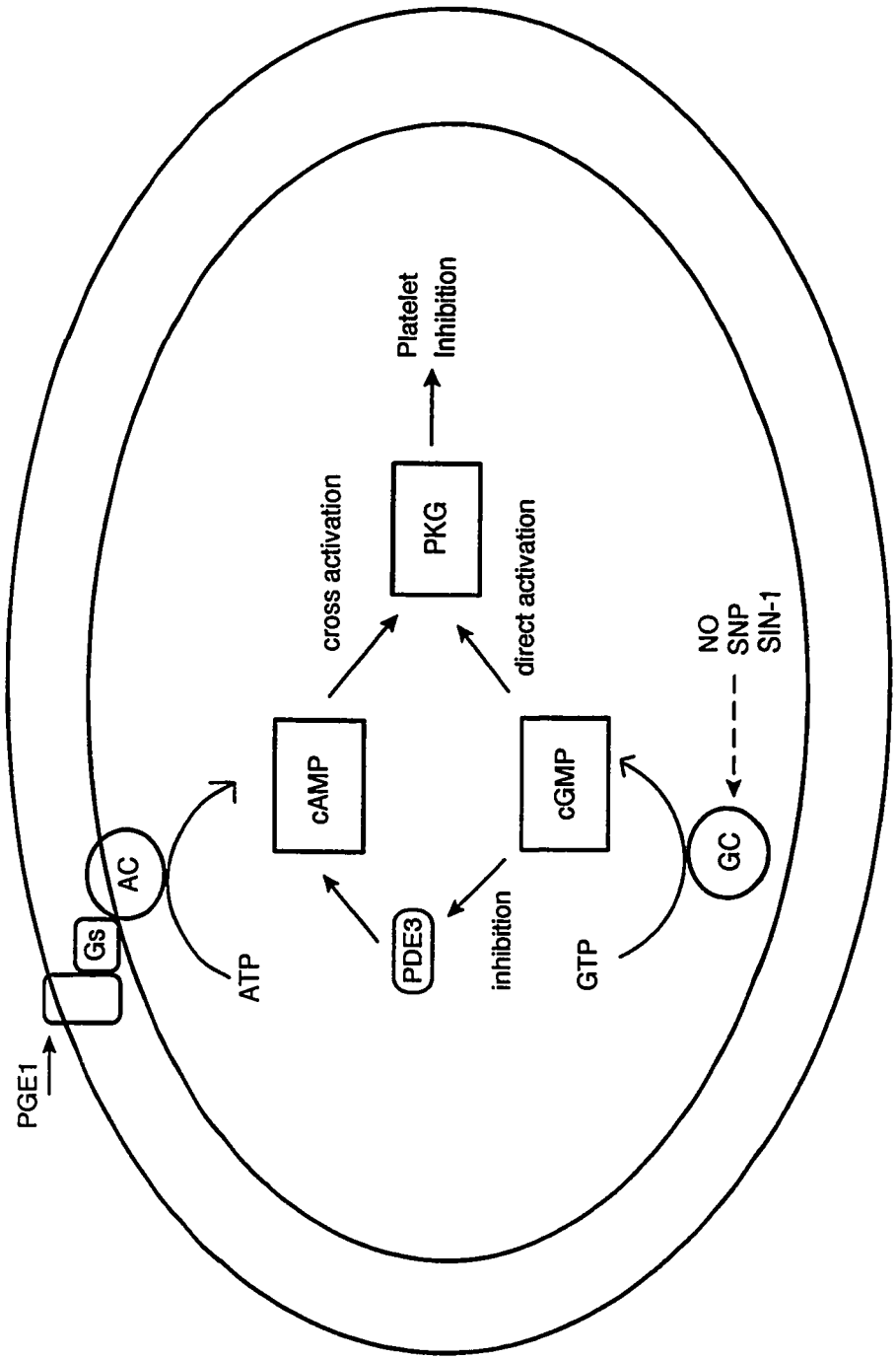
Since PDE3 is responsible for more than 80% of cAMP hydrolysis at submicromolar concentrations of cAMP, and can be negatively regulated by cGMP, it was always possible that cGMP might contribute to increases in intracellular cAMP and platelet inhibition by blocking PDE3 activity. Indeed, this laboratory was the first to establish that inhibition of PDE3 by cGMP mediates the ability of adenylyl and guanylyl cyclase activators to act synergistically to inhibit the aggregation and degranulation of rabbit platelets (Maurice and Haslam, 1990a; Bowen and Haslam, 1991). Maurice and Haslam demonstrated that nitrovasodilators (SNP and SIN-1) caused dose-dependent increases in platelet cAMP, as well as cGMP. In addition, SNP greatly enhanced cAMP accumulation caused by PGE_1 and potentiated the inhibition

of platelet aggregation and degranulation, indicating that nitrovasodilators function to enhance the effects of adenylyl cyclase activators. The AC inhibitor, DDA, was shown to attenuate the increases in cAMP, as well as the corresponding inhibition of platelet aggregation, indicating that the synergistic effects of SNP and PGE_1 were mediated by cAMP. By using a selective inhibitor for PDE3 (cilostamide), these authors observed increases in cAMP levels that were similar to those induced by SNP. Moreover, the increases in cAMP caused by 10 μM cilostamide could not be further enhanced by the addition of SNP, indicating that the effect of SNP on platelet cAMP is through the inhibition of PDE3. Thus, the molecular mechanism by which activators of adenylyl and guanylyl cyclases act synergistically to inhibit platelet function relies to a large degree on the inhibition of PDE3 (see figure 1).

Similar studies were also conducted by Fisch et al. (1995) using human platelets. These investigators likewise measured changes in platelet cyclic nucleotide levels in response to adenylyl and guanylyl cyclase activators. As expected, the combination of SIN-1 with PGE_1, or with iloprost, markedly increased platelet cAMP accumulation. However, the increase in intracellular cAMP levels brought about by higher concentrations of iloprost or PGE_1 appeared to be inhibited by SIN-1. The mechanism by which SIN-1 decreases the cAMP accumulation caused by higher concentrations of prostaglandins was not defined. However, recent results from this laboratory indicate that the activation of PDE2 by cGMP is responsible (see Section 4.3.1) (Dickinson et al., 1996).

Figure 1 Model of contribution made by PDE3 to Platelet inhibition

This diagram illustrates the role played by PDE3 in the inhibition of platelet activation. Increases in cGMP resulting from the actions of nitrovasodilators can increase platelet cAMP by inhibiting PDE3. The elevated levels of cAMP can subsequently cross activate PKG and inhibition platelet function.


PGE1
Gs
AC
ATP
cAMP
PDE3
inhibition
cross activation
PKG
Platelet
Inhibition
direct activation
cGMP
GTP
GC
NO
SNP
SIN-1

Studies conducted with de-endothelialized rat aortic rings showed that SNP and SIN-1 enhanced the ability of isoproterenol to relax precontracted aortic smooth muscle (Maurice et al., 1991). In addition, the combination of these compounds added prior to the contractile stimulus elicited a potent synergistic inhibition of smooth muscle contraction. The synergistic interaction between nitrovasodilators and isoproterenol in rat vascular smooth muscle is analogous to that seen in platelets between nitrovasodilators and PGE_1 or PGI_2. In platelets, this synergism is due to inhibition by cGMP of cAMP hydrolysis by PDE3 (Maurice and Haslam, 1990a). Since PDE3 is present in rat aorta (Lindgren et al., 1990), a similar mechanism of PDE3 inhibition is likely to account for the observed synergism between nitrovasodilators and isoproterenol in this tissue. Indeed, a combination of low concentrations of both SNP and isoproterenol has been shown to increase cAMP accumulation 4 - 5 fold above that seen with isoproterenol alone in these tissues (Maurice and Haslam, 1990b).

More recently, studies conducted by Lugnier and colleagues, suggested that PDE3 and PDE4 function in a cooperative manner to regulate vascular cAMP content and vasodilatation (Lugnier and Komas, 1993; Eckly and Lugnier, 1994). These authors examined the effects of selective PDE inhibitors on relaxation of rat aorta in the presence or absence of a functional endothelium. As expected, PDE3 inhibitors (milrinone, SK&F 94120) effectively induced relaxation of aorta with or without endothelium. In contrast, PDE4 inhibitors (rolipram) required a functional endothelium in order to induce vascular relaxation. Moreover, this relaxation could be

markedly enhanced by the presence of a PDE3 inhibitor or by elevation of cGMP. Measurement of intracellular cAMP content in vascular smooth muscle indicates that low concentrations of PDE3 inhibitors can potentiate the effect of PDE4 inhibitors on cAMP accumulation (Eckly and Lugnier, 1994). Thus, these authors also contend that cGMP enhances cAMP-mediated relaxation by inhibition of PDE3.

Vasorelaxation of rat aorta by the calcitonin gene-related peptide is endothelium-dependent and involves elevations of both cAMP and cGMP (Wang et al., 1991). Moreover, inhibition of NO synthesis from endothelial cells was demonstrated to block vasorelaxation and cyclic nucleotide elevation, suggesting that NO played a role not only in cGMP elevation, but also in cAMP accumulation (Fiscus et al., 1994). Although the increases in cAMP could result from the inhibition of PDE3 by cGMP, no evidence for this possibility was presented. Recently, other investigators, using a porcine pial artery model, demonstrated a correlation between vasodilation, NO release and production of cGMP and cAMP (Armstead, 1995). Thus, PDE3 has emerged as an important intracellular target of cGMP in vascular smooth muscle, as well as in platelets.

5. Thesis objectives

cGMP is a key second messenger in the regulation of platelet adhesion, aggregation and secretion by many physiological and pharmacological agents. Although the direct activation of PKG by cGMP was known to play an important role in the regulation of platelet function at the time the work on which this thesis is based

started (Walter, 1989), there was accumulating evidence that cGMP could also exert major effects by interacting with other important proteins such as cGMP-inhibited phosphodiesterase (PDE3) (Maurice and Haslam, 1990a). To develop a better understanding of the multiple modes of action of cGMP, it was necessary to identify the molecular targets of cGMP. To this end, the first objective of this thesis was to develop a photoaffinity-labelling technique for cGMP-binding proteins, with a view to detecting novel targets of cGMP in platelets and other cells. The results of this work provided further evidence that PDE3 is indeed a major target of cGMP in platelets and led to my second objective, namely to explore the extent to which inhibition of the photolabelling of PDE3 by [^{32}P]cGMP could be used to investigate the effects of drugs on this enzyme without the need for enzyme purification. Since PDE3 is responsible for a major part of cAMP hydrolytic activity in platelets, and increases in cAMP have an inhibitory effect on platelet activation (see section 1.4.1), a great deal of attention has been directed toward PDE3 as a target for potential antithrombotic drugs. Thus, my third major objective was to investigate the molecular mechanisms by which cGMP and inhibitory drugs exert their effects on PDE3. I therefore utilized various molecular biological approaches (cloning, expression and mutagenesis) to characterize further the roles of different domains of the enzyme in the actions of cGMP and selective inhibitors.

CHAPTER 2

PUBLISHED MANUSCRIPT #1

Tang, K. Mary, Judith L. Sherwood and Richard J. Haslam. (1993) Photoaffinity labelling of cyclic GMP-binding proteins in human platelets. *Biochem. J.* 294:329-333.

PREAMBLE

The objective of this work was to optimize the photoaffinity labelling of platelet extracts to detect as many known and novel cGMP-binding proteins as possible. Some of the photolabelled species were identified by immunoprecipitation and sensitivity of photolabelling to selective inhibitors.

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DATE

Photoaffinity labelling of cyclic GMP-binding proteins in human platelets

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Department of Pathology, McMaster University, Hamilton, Ontario, Canada L8N 3Z5

The photoaffinity labelling of platelet cyclic GMP (cGMP)-binding proteins by [^{32}P]cGMP was studied; at least five labelled proteins (110, 80, 55, 49 and 38 kDa) were detected in platelet cytosol and four (80, 65, 49 and 38 kDa) in platelet membranes. The 110 kDa species was identified as cGMP-inhibited cyclic AMP (cAMP) phosphodiesterase (PDE III) by immunoprecipitation and by the inhibition of photolabelling by specific inhibitors of this enzyme. Similarly, the 80 kDa species was identified as cGMP-dependent protein kinase by immunoprecipitation and by the effects of cGMP analogues on photolabelling. Addition of cAMP greatly enhanced the labelling of this 80 kDa protein, implying the existence of a potentially important interaction between the effects of cGMP and cAMP. The 65 kDa photolabelled protein appears to be a novel platelet cyclic-nucleotide-binding protein. In contrast, the 49 and 55 kDa photolabelled species are probably the RI and RII regulatory subunits of cAMP-dependent protein kinase, and the 38 kDa protein(s) may be proteolytic fragment(s) of RI and/or RII.

INTRODUCTION

Cyclic GMP (cGMP) is believed to mediate the inhibition of blood platelet function by nitric oxide and drugs that release nitric oxide [1]. Although direct activation of cGMP-dependent protein kinase cGMP-PK is likely to play a major role in this action of cGMP [2,3], there is increasing evidence that cGMP can also exert inhibitory effects in platelets by preventing the breakdown of cyclic AMP (cAMP) by the cGMP-inhibited cAMP phosphodiesterase (PDE III) [4]. In addition, cGMP exerts major effects in some cells through activation of cGMP-stimulated cyclic nucleotide phosphodiesterases (PDE II) [5] or by direct modulation of ion channels [6,7]. These considerations, and the possible existence of as-yet-unidentified targets for cGMP, indicate a need for a general method of labelling cGMP-binding proteins in cell extracts.

Direct photoaffinity labelling of unidentified cellular proteins by [^{3}H]cGMP has been documented and apparently resulted from photoactivation of aromatic amino acids, rather than of a cGMP [8,9]. In experiments with [^{32}P]cGMP, Groppi et al. [10] observed photoaffinity labelling of the RI subunit of cAMP-dependent protein kinase and of PDE III in K30a lymphoma cells, which overexpress the latter enzyme. In addition, labelling of two platelet proteins of 93 kDa and 78 kDa has been reported in abstract form [11]. Apart from these studies, photoaffinity labelling by [^{32}P]cGMP appears to have been used exclusively to label purified cGMP-binding proteins, specifically PDE II [12,13] and the cGMP-binding cGMP phosphodiesterase (PDE V) [14]. The first objective of the present work was thus to optimize the photoaffinity labelling of platelet cytosolic and membrane proteins by [^{32}P]cGMP, so as to detect as many known and novel cGMP-binding proteins as possible. We then investigated factors that affected the photoaffinity labelling of individual proteins and identified some of these labelled species.

EXPERIMENTAL

Materials

[^{32}P]cGMP (1500 Ci/mmol) was obtained from ICN Biomedicals Canada Ltd. (Mississauga, ON, Canada). Heparin, leupeptin, phenylmethanesulphonyl fluoride, Protein A–Sepharose, cGMP, cAMP, 8-bromo-cGMP, 3-isobutyl-1-methylxanthine (IBMX), Nonidet P40 and protein standards (myosin heavy chain, 205 kDa; β-galactosidase, 116 kDa; phosphorylase *b*, 97.4 kDa; BSA, 66 kDa; ovalbumin, 45 kDa; glyceraldehyde-3-phosphate dehydrogenase, 36 kDa; carbonic anhydrase, 29 kDa) were obtained from Sigma (St. Louis, MO, U.S.A.). Rp-8-bromo-cGMPS, Sp-cAMPS and Rp-cAMPS (see the abbreviations footnote for definition) were supplied by the BioLog Life Science Institute (La Jolla, CA, U.S.A.). Samples of bovine lung cGMP-PK (type 1α) and of a rabbit polyclonal antibody to this protein were generously provided by Dr. U. Walter (Medizinische Universitätsklinik, Würzburg, Germany). Cilostamide was a gift from Professor H. Hidaka (Nagoya University, Nagoya, Japan). Zaprinast (M&B 22948) was from May & Baker (Dagenham, Essex, U.K.) and milrinone from Sterling Drug Inc. (Rensselaer, NY, U.S.A.).

Preparation of platelet cytosol and membranes

Human platelets were isolated and resuspended in Ca^{2+}-free Tyrode's solution (37 °C) containing 5 mM Pipes (buffered to pH 6.5 with NaOH), 50 units of heparin/ml and 75 μg of apyrase/ml by modification of the method of Mustard et al. [15]. After centrifugation (1000 *g* for 15 min), the platelets were finally resuspended at 2.5×10^{9}/ml in a solution containing 100 mM KCl, 25 mM Hepes (buffered to pH 7.4 with NaOH), 2.5 mM EGTA and 0.2 mM leupeptin (solution A). The suspension was

Abbreviations used: cGMP, cAMP, cyclic GMP and cyclic AMP respectively; PDE III, cGMP-inhibited cAMP phosphodiesterase; RI and RII, regulatory subunits of cAMP-dependent protein kinase; cGMP-PK, cGMP-dependent protein kinase; IBMX, 3-isobutyl-1-methylxanthine; Rp-8-bromo-cGMPS, 8-bromoguanosine cyclic 3',5'-monophosphorothioate, Rp isomer; Sp-cAMPS, adenosine cyclic 3',5'-monophosphorothioate, Sp isomer; Rp-cAMPS, adenosine cyclic 3',5'-monophosphorothioate, Rp isomer; PDE II, cGMP-stimulated cyclic nucleotide phosphodiesterase; PDE V, cGMP-binding cGMP phosphodiesterase.

* To whom correspondence should be addressed.

then frozen in methanol/solid CO_2 and stored at −70 °C. Platelets were then lysed by freezing and thawing twice, and the lysate was centrifuged at 40000 *g* for 40 min to isolate the membrane fraction, which was resuspended in solution A. The protein concentrations in the supernatant (cytosol) and membrane fractions were determined [16] and adjusted to 1 mg/ml.

Photoaffinity labelling of platelet proteins by [^{32}P]cGMP

Incubation mixtures (100 μl) containing 75–80 μl of platelet cytosol or membrane suspension and 20–25 μl of additions were placed in the wells of a Microtest III 96-well tissue-culture plate (Falcon 3072) resting in an ice tray. Additions were dissolved in dimethyl sulphoxide (final concn. 0.2 %, v/v) or in solution A and included 5 μCi of [^{32}P]cGMP (30–60 nM after dilution) and dithiothreitol (1 mM after dilution). Nickel-wire stir bars were added to each well. After 15 min equilibration in the dark, the stirred samples were irradiated for a further 15 min with a 30 W germicidal lamp (Westco, Hamilton, ON, Canada) placed 5 cm above the open plate. The irradiated samples received 1.1 mW/cm^2 (J-225 u.v. meter; Ultraviolet Products Inc., San Gabriel, CA, U.S.A.). This experimental design permitted simultaneous labelling of multiple samples in a reproducible manner. A 90 μl portion of each sample was then mixed with 0.5 ml of 10 % (w/v) trichloroacetic acid. After 30 min, the precipitated protein was isolated by centrifugation, dissolved in electrophoresis sample buffer and analysed by SDS/PAGE (usually with 10 % acrylamide) [17]. Dried gels were exposed overnight on Kodak X-OMAT AR film.

Preparation of anti-PDE III antiserum

A synthetic peptide corresponding to the C-terminal 14 residues of human platelet PDE III [18] was synthesized and conjugated to rabbit serum albumin by Multiple Peptide Systems (San Diego, CA, U.S.A.). Male New Zealand White rabbits were given multiple intradermal injections of peptide conjugate (1 mg of peptide/rabbit) mixed with Freund's complete adjuvant. After four booster injections of conjugate mixed with Freund's incomplete adjuvant, the rabbits were bled out and sera were evaluated by immunoblotting of platelet proteins; a serum that at 1:1000 dilution gave a 110 kDa signal that was blocked by 10 μM immunizing peptide was selected.

Immunoprecipitation

Samples (100 μl) containing photoaffinity-labelled proteins were diluted to 1 ml with a solution containing 50 mM Tris/HCl, pH 7.5, 100 mM NaCl, 1 % (v/v) Nonidet P40 and 0.5 % (w/v) sodium deoxycholate (solution B) supplemented with 1 mM phenylmethanesulphonyl fluoride, 0.25 mM phenanthroline and 0.2 mM leupeptin. Any insoluble proteins were removed by centrifugation at 100000 *g* for 30 min. Both the cytosolic and soluble membrane proteins were pre-cleared twice with heat-killed formaldehyde-fixed *Staphylococcus aureus* (i.e. rocked for 2 h at 4 °C). Each of the labelled protein fractions was then incubated for 1 h at 0 °C with 5 μl of antiserum, followed by agitation for 2 h at 4 °C with 50 μl of 50 % Protein A–Sepharose. The Protein A–Sepharose was isolated by centrifugation (12000 *g*, 1 min), washed three times with solution B, resuspended in electrophoresis sample buffer [17] and boiled for 5 min before analysis by SDS/PAGE.

RESULTS

Factors affecting photoaffinity labelling of platelet proteins by [^{32}P]cGMP

Preincubation and u.v. irradiation of a mixture of platelet cytosol fraction and [^{32}P]cGMP (see the Experimental section) led to a selective incorporation of ^{32}P into 110, 55, 49 and 38 kDa proteins, as well as to less marked labelling of a few other proteins (100, 85 and 80 kDa) (Figure 1a). Both the 49 kDa and 38 kDa proteins appeared as closely spaced doublets in briefly exposed autoradiographs. Irradiation was essential for the labelling of all species, except the cytosolic 38 kDa proteins (Figure 1a, lane 1). In platelet membrane fractions, less ^{32}P was incorporated into the 110 kDa protein and much more into the 80 kDa species (Figure 1b). In addition, photolabelled membranes contained a 65 kDa labelled protein not found in cytosol, a 49 kDa protein corresponding to that in the cytosol (but no 55 kDa species) and a 38 kDa protein that co-electrophoresed with the upper cytosolic component.

Addition of $MgCl_2$ (5 mM) exerted differential effects on the labelling of these proteins, enhancing that of most species, but diminishing that of the 80 kDa protein (Figure 1). In view of possible losses of [^{32}P]cGMP by enzymic hydrolysis, we studied the effects of zaprinast, an inhibitor of cGMP phosphodiesterase [19]. At 10 μM, this compound enhanced photolabelling of some of the above proteins in both the presence and absence of $MgCl_2$, and particularly that of the 110 kDa species (Figure 1). both $MgCl_2$ and zaprinast were therefore used in the subsequent experiments that focused on the cytosolic 110 kDa protein, whereas zaprinast alone was used in experiments on platelet membranes and the 80 kDa protein. In the presence of these additions, increases in the amounts of cytosolic protein (10–80 μg) and of [^{32}P]cGMP (1–8 μCi) were associated with corresponding increases in ^{32}P incorporation into the above-

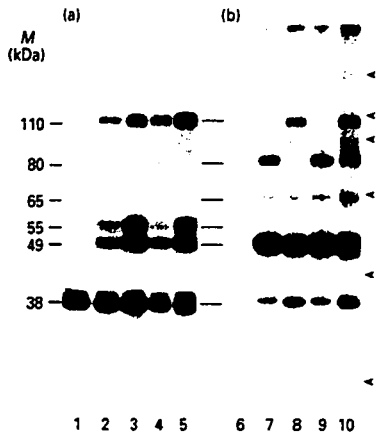

Figure 1 Effects of Mg^{2+} and zaprinast on the photoaffinity labelling of human platelet cytosolic and membrane proteins by [^{32}P]cGMP

Samples containing platelet cytosol (lanes 1–5) and membranes (lanes 6–10) were incubated with [^{32}P]cGMP at 0 °C. Protein from lanes 1 and 6 was not irradiated, whereas that in lanes 2–5 and 7–10 was irradiated for 15 min at 0 °C. Other additions were: lanes 1, 2, 6 and 7, none; lanes 3 and 8, 5 mM $MgCl_2$; lanes 4 and 9, 10 μM zaprinast; lanes 5 and 10, 5 mM $MgCl_2$ and 10 μM zaprinast. Protein was analysed by SDS/PAGE; an autoradiograph is shown. The positions of standard proteins (see the text) are shown on the right (◄) and the calculated molecular masses (*M*) of labelled proteins on the left.

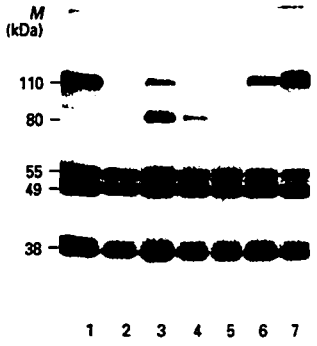

Figure 2 Effects of various compounds on the photoaffinity labelling of human platelet cytosolic proteins by [^{32}P]cGMP

All samples contained 5 mM $MgCl_2$ and 10 μM zaprinast. Other conditions (all 10 μM) were: lane 1, none; lane 2, cGMP; lane 3, cAMP; lane 4, cilostamide; land 5, milrinone; lane 6, IBMX; lane 7, GMP. After irradiation, protein from each sample was analysed by SDS/PAGE (13% acrylamide); an autoradiograph is shown. The calculated molecular masses (*M*) of the labelled proteins are shown on the left.

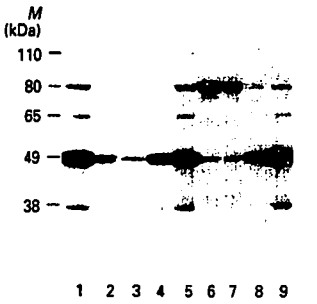

Figure 3 Effects of various compounds on the photoaffinity labelling of human platelet membrane proteins by [^{32}P]cGMP

All samples contained 10 μM zaprinast, but no $MgCl_2$. Other additions (all 20 μM) were: lane 1, none; lane 2, cGMP; lane 3, 8-bromo-cGMP; lane 4, Rp-8-bromo-cGMPS; lane 5, GMP; lane 6, cAMP; lane 7, Sp-cAMPS; lane 8, Rp-cAMPS; lane 9, AMP. After irradiation, protein from each sample was analysed by SDS/PAGE; an autoradiograph is shown. The calculated molecular masses (*M*) of the labelled proteins are indicated on the left.

labelled species. We used 80 μg of protein and 5 μCi of [^{32}P]cGMP in most experiments, as this gave strong signals after autoradiography overnight.

Effects of competing compounds on photoaffinity labelling by [^{32}P]cGMP

We compared the effects of 10 μM concentrations of various compounds on the photolabelling of platelet cytosolic proteins by [^{32}P]cGMP (Figure 2). Unlabelled cGMP decreased the labelling of all the proteins studied, but particularly that of the 110 kDa species. However, cAMP only inhibited labelling of the latter protein and, unexpectedly, induced marked labelling of the 80 kDa species. Photolabelling of the 110 kDa protein was partially inhibited by IBMX and potently inhibited by the specific PDE III inhibitors, cilostamide and milrinone [20], which

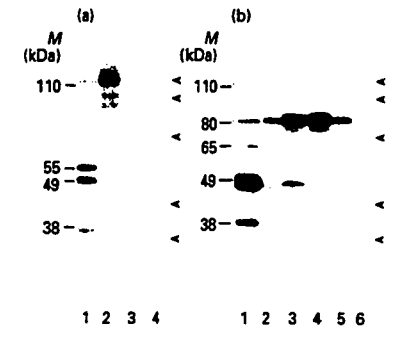

Figure 4 Immunoprecipitation of photoaffinity-labelled platelet proteins using antisera to platelet PDE III and to bovine lung cGMP-PK

(**a**) Human platelet cytosol was photoaffinity-labelled with [^{32}P]cGMP in the presence of 5 mM $MgCl_2$ and 5 μM zaprinast. Control labelled protein (33 μg) and immunoprecipitates from 80 μg of labelled protein were analysed by SDS/PAGE; an autoradiograph is shown. Lane 1, control; lane 2, immunoprecipitate using anti-PDE III antiserum; lane 3, immunoprecipitate using anti-PDE III antiserum plus 5 μM immunizing peptide; lane 4, immunoprecipitate using preimmune serum. (**b**) human platelet membranes were photoaffinity labelled with [^{32}P]cGMP in the presence of 10 μM zaprinast alone (lanes 1 and 2) or of 10 μM zaprinast and 10 μM cAMP (lanes 3–6). Control labelled protein (53 μg) and immunoprecipitates from 80 μg of membrane protein were analysed by SDS/PAGE; an autoradiograph is shown. Lanes 1 and 3, controls; lanes 2 and 4, immunoprecipitates using anti-cGMP-PK antiserum; lane 5, immunoprecipitate using anti-cGMP-PK antiserum plus cGMP-PK (0.8 μg); lane 6, immunoprecipitate using non-immune serum. The positions of standard proteins and the calculated molecular masses (*M*) of the labelled proteins are shown.

had only weak effects on the labelling of other proteins. GMP had no effect on labelling of the 110 kDa protein, but did to some extent inhibit labelling of the 55, 49 and 38 kDa species. AMP (20 μM) had no significant effects (results not shown).

In similar experiments with platelet membranes (Figure 3), 20 μM concentrations of cGMP, 8-bromo-cGMP and Rp-8-bromo-cGMPS inhibited photolabelling of the 80, 65, 49 and 38 kDa proteins, whereas the same concentration of GMP had no such effects. At 20 μM, either cAMP or Sp-cAMPS markedly increased labelling of the 80 kDa protein, though these two compounds inhibited labelling of all other proteins (Figure 3). An additional 76 kDa species, presumably related to the 80 kDa protein, was usually detected when labelling of the latter was highly stimulated by cAMP or Sp-cAMPS. In contrast with Sp-cAMPS, 20 μM Rp-cAMPS decreased labelling of the 80 kDa protein, in addition to inhibiting labelling of the 65, 49 and 38 kDa species. AMP (20 μM) had no effects on the photolabelling of any of these membrane proteins.

Identification of the 110 kDa and 80 kDa proteins

By using a rabbit antiserum to PDE III, we were able to immunoprecipitate the 110 kDa photolabelled protein from platelet cytosol (Figure 4a). The only other labelled proteins in the immunoprecipitate were the minor 100 kDa and 85 kDa species. Addition of immunizing peptide prevented immunoprecipitation of labelled proteins, and none were seen when preimmune serum was used. We also selectively immunoprecipitated the 80 kDa photolabelled protein from detergent-solubilized platelet membrane fraction, using an antiserum to bovine lung cGMP-PK (Figure 4b) When cAMP was added to enhance photolabelling, the 76 kDa species was immunoprecipitated with the 80 kDa protein. Immunoprecipitation of

Figure 5 Photoaffinity labelling of purified bovine lung cGMP-PK by [^{32}P]cGMP: effects of platelet membranes and cAMP

Samples containing cGMP-PK (0.4 μg) (lanes 1 and 6), membranes (75 μg of protein) (lanes 2 and 4) or both cGMP-PK and membranes (lanes 3 and 5) were photoaffinity-labelled in the presence of 10 μM zaprinast (lanes 1–3) or of 10 μM zaprinast and 10 μM cAMP (lanes 4–6). The labelled protein was analysed by SDS/PAGE; an autoradiograph is shown. The positions of standard proteins are shown on the right and the calculated molecular masses (M) of the labelled proteins on the left. The ^{32}P in the 80 kDa labelled protein was determined (407, 88, 1321, 268, 2114 and 132 c.p.m. in lanes 1–6 respectively).

these proteins was decreased by purified bovine lung cGMP-PK and was not seen when non-immune serum was used.

To confirm that the 80 kDa labelled protein was platelet cGMP-PK, we photolabelled a sample of the purified 76 kDa type Iα enzyme from bovine lung and obtained a labelled species that migrated at 80 kDa on SDS/PAGE (Figure 5, lane 1). In the absence of membranes, cAMP inhibited photoaffinity labelling of purified cGMP-PK (Figure 5, lane 6). However, photolabelling of the enzyme was greatly enhanced by the addition of membranes in the absence or presence of cAMP (Figure 5, lanes 3 and 5). The total photolabelling obtained with a mixture of 75 μg of membrane protein and 0.4 μg of purified cGMP-PK was 2.5–5-fold greater than the sum of that seen when these components were labelled individually.

DISCUSSION

Human platelets contain a cGMP-inhibited cAMP phosphodiesterase (PDE III) with an apparent subunit molecular mass on SDS/PAGE of 110 kDa [20]. Two lines of evidence indicate that the 110 kDa protein photolabelled by [^{32}P]cGMP corresponds to this enzyme. First, photoaffinity labelling of this species was blocked not only by cGMP and cAMP, but also by specific PDE III inhibitors, including cilostamide and milrinone. Second, the labelled protein was selectively immunoprecipitated by an antibody to PDE III. Although an early study showed that PDE III could be photolabelled by [^{32}P]cGMP in K30a lymphoma cells, which overexpress this enzyme [10], PDE III has not previously been detected by this method in platelets. This enzyme is readily proteolysed [20], and it is likely that the minor 85 and 100 kDa photolabelled species found in platelet cytosol, which were also immunoprecipitated by PDE III antiserum, are proteolytic fragments of this enzyme. It is also possible that PDE III and these fragments conceal some cGMP-stimulated phosphodiesterase (PDE II), which has an apparent molecular mass of 102–105 kDa [19] and is present in small amounts in platelets [21]. The 84–99 kDa cGMP-specific phosphodiesterase (PDE V) [19], which is a major cGMP-binding species in platelets [22], was not observed in our experiments, despite a preliminary report that it can be photolabelled in platelets [11]. This discrepancy was not attributable to the addition of zaprinast. Also, we have shown (K. M. Tang, J. L. Sherwood and R. J. Haslam, unpublished work) that it is not due to the omission of IBMX, which potentiates the binding of cGMP to this enzyme [22]. However, a higher pH than was used in the present experiments is optimal for cGMP binding to platelet PDE V [22,23].

Several lines of evidence indicate that the 80 kDa photolabelled protein is the platelet cGMP-PK [2]. First, these proteins display the same subcellular distribution. Secondly, the 80 kDa labelled protein co-migrated with the purified photolabelled bovine lung (type Iα) enzyme. Thirdly, antibody to the bovine lung cGMP-PK immunoprecipitated the 80 kDa photolabelled protein. Finally, cGMP analogues that bind to cGMP-PK, including its 8-bromo- and 3',5'-monophosphorothioate derivatives [24,25], inhibited photolabelling. In contrast, cAMP and Sp-cAMPS greatly stimulated labelling of the 80 kDa protein, though the Rp-cAMPS diastereomer was inhibitory. A similar effect of cAMP on the photolabelling of a platelet membrane protein was briefly reported by Walseth et al. [11], although the labelled species was not identified. Since cAMP did not stimulate photolabelling of the purified bovine lung enzyme, and both diastereomers of cAMPS have been shown to inhibit binding of cGMP to this protein [26], it seemed possible that autophosphorylation or cAMP-dependent phosphorylation of the membrane cGMP-PK might be responsible for the effect observed in membranes. Although a slight decrease in the electrophoretic mobility of cGMP-PK photolabelled in the presence of cAMP (Figure 5) is consistent with this possibility, no other evidence for phosphorylation of this enzyme was obtained in preliminary experiments. Indeed, autophosphorylation of cGMP-PK has been reported to enhance cAMP binding [27], which should decrease photolabelling. Addition of platelet membranes to purified cGMP-PK with or without cAMP greatly facilitated photolabelling of this enzyme. Although the factors in platelet membranes responsible for these effects have not been identified, our results indicate that cAMP may be able to increase cGMP binding to platelet cGMP-PK, and suggest the possibility of a novel co-operative interaction between the effects of these cyclic nucleotides.

The 65 kDa platelet membrane protein that was photoaffinity-labelled does not correspond to any known platelet cGMP-binding protein. This protein is unlikely to be a proteolytic fragment of cGMP-PK, since it was not immunoprecipitated with this enzyme and its labelling was affected differently by Mg^{2+}. Since photolabelling of the 65 kDa protein was inhibited by both cyclic nucleotides, it is possible that it represents a novel membrane site of action of these compounds. It may be relevant that the apparent molecular mass of the subunit of the retinal rod cGMP-gated ion channel is 63 kDa and that similar cyclic-nucleotide-gated ion channels exist in other cell types [6,7,28].

It is likely that the 49 and 55 kDa platelet proteins photolabelled by [^{32}P]cGMP are the RI and RII regulatory subunits of cAMP-dependent protein kinase, which migrate more slowly on SDS/PAGE than expected from their true molecular masses [29]. Thus, these photolabelled proteins had molecular masses and subcellular distributions identical with those previously observed after photoaffinity labelling of platelet RI and RII by 8-azido-cAMP [30]. Photolabelling of RI by [^{32}P]cGMP has also been observed in K30a lymphoma cells. The 38 kDa group of labelled proteins may be proteolytic fragments of the RI and/or RII regulatory subunits, since species of this size were also observed after photoaffinity labelling with 8-azido-cAMP and were increased in amount after Ca^{2+}-dependent proteolysis [30].

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REFERENCES

1 Walter, U. (1989) Rev. Physiol. Biochem. Pharmacol. **113**, 41–88
2 Waldmann, R., Bauer, S., Göbel, C., Hoffmann, F., Jakobs, K. H. and Walter, U. (1986) Eur. J. Biochem. **158**, 203–210
3 Geiger, J., Nolte, C., Butt, E., Sage, S. O. and Walter, U. (1992) Proc. Natl. Acad. Sci. U.S.A. **89**, 1031–1035
4 Maurice, D. H. and Haslam, R. J. (1990) Mol. Pharmacol. **37**, 671–681
5 MacFarland, R. T., Zelus, B. D. and Beavo, J. A. (1991) J. Biol. Chem. **266**, 136–142
6 Kaupp, U. B. (1991) Trends Neurosci. **14**, 150–157
7 Light, D. B., Corbin, J. D. and Stanton, B. A. (1990) Nature (London) **344**, 336–339
8 Antonoff, R. S., Ferguson, J. J. and Idelkope, G. (1976) Photochem. Photobiol. **23**, 327–329
9 Antonoff, R. S. and Ferguson, J. J. (1978) Photochem. Photobiol. **27**, 499–501
10 Groppi, V. E., Steinberg, F., Kaslow, H. R., Walker, N. and Bourne, H. R. (1983) J. Biol. Chem. **258**, 9717–9723
11 Walseth, T. F., Yuen, P. S. T., Panter, S. S. and Goldberg, N. D. (1985) Fed. Proc. Fed. Am. Soc. Exp. Biol. **44**, 728
12 Stroop, S. D., Charbonneau, H. and Beavo, J. A. (1989) J. Biol. Chem. **264**, 13718–13725
13 Murashima, S., Tanaka, T., Hockman, S. and Manganiello, V. (1990) Biochemistry **29**, 5285–5292
14 Thomas, M. K., Francis, S. H. and Corbin, J. D. (1990) J. Biol. Chem. **265**, 14964–14970
15 Mustard, J. F., Perry, D. W., Ardlie, N. G. and Packham, M. A. (1972) Br. J. Haematol. **22**, 193–204
16 Lowry, O. H., Rosebrough, N. J., Farr, A. L. and Randall, R. J. (1951) J. Biol. Chem. **193**, 265–275
17 Laemmli, U. K. (1970) Nature (London) **227**, 680–685
18 Meacci, E., Taira, M., Moos, M., Smith, C. J., Movsesian, M. A., Degerman, E., Belfrage, P. and Manganiello, V. (1992) Proc. Natl. Acad. Sci. U.S.A. **89**, 3721–3725
19 Thompson, W. (1991) Pharmacol. Ther. **51**, 13–33
20 MacPhee, C. H., Harrison, S. A. and Beavo, J. A. (1986) Proc. Natl. Acad. Sci. U.S.A. **83**, 6660–6663
21 Grant, P. G., Mannarino, A. F. and Colman, R. W. (1990) Thromb. Res. **59**, 105–119
22 Coquil, J.-F., Franks, D. J., Wells, J. N., Dupuis, M. and Hamet, P. (1980) Biochim. Biophys. Acta **631**, 148–165
23 Robichon, A. (1991) J. Cell. Biochem. **47**, 147–157
24 Butt, E., Nolte, C., Schulz, S., Beltman, J., Beavo, J. A., Jastorff, B. and Walter, U. (1992) Biochem. Pharmacol. **43**, 2591–2600
25 Butt, E., van Bemmelen, M., Fischer, L., Walter, U. and Jastorff, B. (1990) FEBS Lett. **263**, 47–50
26 Hofmann, F., Gensheimer, H.-P., Landgraf, W., Hullin, R. and Jastorff, B. (1985) Eur. J. Biochem. **150**, 85–88
27 Landgraf, W., Hullin, R., Göbel, C. and Hofmann, F. (1986) Eur. J. Biochem. **154**, 113–117
28 Ahmad, I., Korbmacher, C., Segal, A. S., Cheung, P., Boulpaep, E. L. and Barnstable, C. J. (1992) Proc. Natl. Acad. Sci. U.S.A. **89**, 10262–10266
29 Takio, K., Smith, S. B., Krebs, E. G., Walsh, K. A. and Titani, K. (1982) Proc. Natl. Acad. Sci. U.S.A. **79**, 2544–2548
30 Salama, S. E. and Haslam, R. J. (1981) FEBS Lett. **130**, 230–234

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CHAPTER 3

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PREAMBLE

In these experiments the inhibitory effects of selected compounds on the photolabelling of unpurified PDE3 were quantitated and compared in platelet cytosol. Crude rat tissue extracts were also photolabelled with [^{32}P]cGMP to demonstrate the general applicability of this technique and to detect previously unknown cGMP-binding proteins.

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Photoaffinity labelling of cyclic GMP-inhibited phosphodiesterase (PDE III) in human and rat platelets and rat tissues: effects of phosphodiesterase inhibitors

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Abstract

Ultraviolet irradation of human platelet cytosol in the presence of ^{32}P-labelled cyclic GMP (cGMP) can specifically label 110, 80, 55, 49 and 38 kDa proteins; the 110 kDa species is the subunit of cGMP-inhibited phosphodiesterase (PDE III) and the 80 kDa species that of cGMP-dependent protein kinase (Tang et al., 1993, Biochem. J. 294, 329). We have now shown that although photolabelling of platelet PDE III was inhibited by unlabelled cGMP, 8-bromo-cGMP and cyclic AMP (cAMP), it was not affected by phosphorothioate analogues of these cyclic nucleotides. Specific concentration-dependent inhibitions of the photolabelling of PDE III were observed with the following PDE inhibitors: trequinsin ($IC_{50} = 13 \pm 2$ nM), lixazinone ($IC_{50} = 22 \pm 4$ nM), milrinone ($IC_{50} = 56 \pm 12$ nM), cilostamide ($IC_{50} = 70 \pm 9$ nM), siguazodan ($IC_{50} = 117 \pm 29$ nM) and 3-isobutyl 1-methylxanthine (IBMX) ($IC_{50} = 3950 \pm 22$ nM). Thus, measurements of the inhibitory effects of compounds on the photolabelling of platelet PDE III provide a simple quantitative means of investigating their actions at a molecular level that avoids the need to purify the enzyme. Photolabelling of rat platelet lysate or rat heart homogenate by [^{32}P]cGMP showed that the 110 kDa PDE III present in human material was replaced by a 115 kDa protein, labelling of which was also blocked by PDE III inhibitors. Heart and other rat tissues contained much less of this putative 115 kDa PDE III than rat platelets. In contrast, the 80 kDa protein was labelled much less in platelets than in many other rat tissue homogenates (e.g., heart, aorta, uterus and lung). Thus, comparison of the relative amounts of specific photolabelled proteins in different cells may provide an indication of different patterns of cyclic nucleotide action. We compared the abilities of phosphodiesterase inhibitors to block the photolabelling of PDE III in human platelet cytosol and to increase the iloprost-stimulated accumulation of cAMP in intact platelets. Whereas trequinsin ($EC_{50} = 19 \pm 3$ nM), lixazinone ($EC_{50} = 122 \pm 8$ nM), milrinone ($EC_{50} = 5320 \pm 970$ nM) and siguazodan ($EC_{50} = 18880 \pm 3110$ nM) all increased platelet cAMP to the same maximum extent, cilostamide and IBMX increased cAMP further, indicating that they inhibited a PDE isozyme in addition to PDE III. Differences in membrane permeability of these compounds may account for discrepancies between their abilities to inhibit photolabelling of PDE III and to increase platelet cAMP.

Key words: Photoaffinity labelling; Cyclic GMP-inhibited phosphodiesterase (PDE III); Platelet; Phosphodiesterase inhibitor; Cyclic AMP

1. Introduction

The major cyclic nucleotide phosphodiesterase responsible for the breakdown of low concentrations of cyclic AMP (cAMP) in platelets is the cyclic GMP (cGMP)-inhibited enzyme, now designated PDE III (Beavo, 1988; Beavo and Reifsnyder, 1990; Thompson, 1991). Since increases in cAMP block the responses of platelets to all aggregating agents (Haslam et al., 1978), this enzyme has attracted interest as a target for potential antithrombotic drugs (Macphee et al., 1986; Alvarez et al., 1986; Seiler et al., 1991; Saitoh et al., 1993). Moreover, inhibition of this enzyme by cGMP plays a role in the actions of nitrovasodilators on platelets (Maurice and Haslam, 1990). Platelet PDE III is now known to be a soluble homodimer containing 110 kDa subunits (Macphee et al., 1986). This enzyme is similar to the cardiac PDE III, which has been widely studied as a receptor for potential cardiotonic

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agents (Harrison et al., 1986; Erhardt et al., 1988; Murray and England, 1992). Complete purification of the intact platelet enzyme has proved very difficult because of its high susceptibility to proteolysis (Grant and Colman, 1984; Macphee et al., 1986) and many authors have therefore used partially purified enzyme to study the effects of inhibitors (e.g., Alvarez et al., 1986; Weishaar et al., 1986; Murray et al., 1990) or have assumed that activities measured in platelet lysate in the presence of submicromolar cAMP concentrations are sufficiently specific (e.g., Seiler et al., 1991). The cardiac PDE III must be separated from cAMP-specific phosphodiesterase (PDE IV) before meaningful studies with inhibitors can be carried out (Reeves et al., 1987).

In an attempt to circumvent these problems and to derive a method of investigating the effects of PDE III inhibitors on this enzyme in different tissues without enzyme purification, we have exploited the ability of [^{32}P]cGMP to photolabel the active centre of the enzyme. Thus, Groppi et al. (1983) reported that in the presence of UV light, [^{32}P]cGMP photolabelled a cyclic nucleotide phosphodiesterase that was overexpressed in a mutant S49 lymphoma cell line (K30a cells). Labelling of this enzyme (106 kDa) was blocked by cGMP, cAMP and 3-isobutyl 1-methylxanthine (IBMX), but not by Ro 20-1724. Subsequently, it became clear that this enzyme is a cGMP-inhibited isoenzyme of PDE (Bourne et al., 1984; Reifsnyder et al., 1985), similar to PDE III from other sources (Beavo, 1988). Recently, we have studied the proteins that are photolabelled by [^{32}P]cGMP in platelet cytosol and membrane fractions and were able to identify a soluble 110 kDa labelled species as PDE III by specific immunoprecipitation (Tang et al., 1993). We were also able to enhance photolabelling of PDE III in platelet cytosol by including Mg^{2+} ions and zaprinast, an inhibitor of cGMP phosphodiesterase, in the medium. In contrast, high concentrations of two PDE III inhibitors, cilostamide and milrinone, blocked photolabelling. These observations suggested that quantitative analysis of the effects of PDE III inhibitors on photolabelling of the unpurified enzyme might be feasible in platelet cytosol. We have now explored this possibility and have compared the results with the effects of the same inhibitors on the accumulation of cAMP in intact platelets. In addition, we have carried out experiments with rat tissues to test the general applicability of this photolabelling technique.

2. Materials and methods

2.1. Materials

[^{32}P]cGMP (1500 Ci/mmol) and [^{14}C]cAMP (54 mCi/mmol) were obtained from ICN Biomedicals Canada (Mississauga, ON, Canada) and [2-^{3}H]adenine (29 Ci/mmol) from DuPont (Mississauga, ON, Canada). *R*p-8-bromoguanosine-3',5'-cyclic monophosphorothioate (*R*p-8-bromo-cGMPS), *S*p-adenosine-3',5'-cyclic monophosphorothioate (*S*p-cAMPS) and *R*p-adenosine-3',5'-cyclic monophosphorothioate (*R*p-cAMPS) were supplied by BioLog Life Science Institute (La Jolla, CA, USA). cAMP, cGMP, 8-bromo-cGMP, IBMX and protein standard solution were from Sigma (St. Louis, MO, USA), as were protein markers for sodium dodecyl sulphate-polyacrylamide gel electrophoresis (SDS-PAGE) (*β*-galactosidase, 116 kDa; phosphorylase *b*, 97.4 kDa; bovine serum albumin, 66 kDa; ovalbumin, 45 kDa; glyceraldehyde-3-phosphate dehydrogenase, 36 kDa; carbonic anhydrase, 29 kDa). The following compounds were gifts: zaprinast (May and Baker, Dagenham, Essex, UK), trequinsin (HL 725) (Hoechst, Frankfurt-am-Main, Germany), lixazinone (Syntex Corporation, Palo Alto, CA, USA), milrinone (Sterling Drug, Rensselaer, NY, USA), siguazodan (SmithKline Beecham, Welwyn, Herts., UK), rolipram and iloprost (Schering, Berlin, Germany) and vinpocetine (Gedeon Richter, Budapest, Hungary). Cilostamide was generously supplied by Professor H. Hidaka (Nagoya University, Nagoya, Japan).

2.2. Preparation of platelet lysates and cytosol fraction for photoaffinity labelling

Human platelets were isolated and washed using a modification (Tang et al., 1993) of the method of Mustard et al. (1972). Rat platelets were prepared using 20% higher centrifugal forces. The platelets were finally suspended at 2.5×10^9/ml in a medium containing 100 mM KCl, 25 mM Hepes (pH 7.4), 2.5 mM EGTA and 0.2 mM leupeptin (solution A) and were then lysed by freezing and thawing twice. When required, the cytosol fraction was isolated by centrifugation at $40{,}000 \times g$ for 40 min at 4°C. The protein in the lysate or cytosol fraction was determined immediately (Lowry et al., 1951) and adjusted to 1 mg/ml with solution A.

2.3. Preparation of rat tissue homogenates

Wistar rats (approx. 250 g) were anaesthetized with pentobarbital (50 mg/kg, i.p.) and then perfused for 10 min through a cannulated carotid artery with Krebs-Henseleit physiological saline containing 1 nM iloprost. By this means, platelets which contain a high concentration of PDE III, were almost completely removed from the tissues, samples of which were then rapidly frozen in liquid N_2. Tissues were stored at −70°C until used, when they were ground to a fine powder and then homogenized in solution A. After

protein determination (Lowry et al., 1951), homogenates were diluted to 1 mg of protein/ml.

2.4. Photoaffinity labelling of proteins by [^{32}P]cGMP

The methods used have been described (Tang et al., 1993). Briefly, incubation mixtures (in a final volume of 100 μl of solution A) contained 75–100 μg of cellular protein, 5 mM $MgCl_2$, 10 μM zaprinast, 33 nM [^{32}P]-cGMP (5 to 2.5 μCi, depending on decay of the ^{32}P) and other additions, as indicated. In some experiments, cellular protein and [^{32}P]cGMP were mixed before addition to other components; in other experiments (indicated in the figure legends), [^{32}P]cGMP was added last. These mixtures were stirred at 0°C for 15 min in the dark, and then for a further 15 min period under UV light (a 30 W germicidal lamp held at a distance of 5 cm). Protein was then precipitated with trichloroacetic acid and analysed by SDS-PAGE (Laemmli, 1970) using, unless stated otherwise, 10% acrylamide. After autoradiography of the dried gels on Kodak X-OMAT AR film, the 110 kDa PDE III was cut from the gels and counted for Čerenkov radiation (Haslam and Lynham, 1977).

2.5. Measurement of increases in [^{3}H]cAMP in intact human platelets

Changes in [^{3}H]cAMP were measured in washed human platelets that had been labelled by preincubation with [^{3}H]adenine (Haslam and Vanderwel, 1989). In this procedure, the platelets were suspended at 3.2×10^9/ml in Tyrode's solution containing 5 mM Hepes (pH 7.35), 0.35% bovine serum albumin and 60 μg of apyrase/ml, and were incubated for 30 min at 37°C with 2 μM [^{3}H]adenine that had been adjusted to a final specific radioactivity of 10 Ci/mmol. After labelling, the platelets were isolated by centrifugation and finally resuspended at 4.44×10^8/ml in the same medium except that the apyrase concentration was reduced to 6 μg/ml. This suspension was stored at 37°C until used (within 1 h). Incubation mixtures (final volume 0.5 ml) contained 0.45 ml of platelet suspension (2×10^8 platelets) and 0.05 ml of additions in 0.154 M NaCl. Compounds with poor solubility in aqueous solution were added in 1 μl of dimethyl sulphoxide (final concentration 0.2% v/v). Incubations were carried out in triplicate at 37°C and were terminated after 0.5 min by addition of 0.5 ml of 20% (w/v) trichloroacetic acid, followed by 1000 d.p.m. of [^{14}C]cAMP to monitor the recovery of [^{3}H]cAMP. Labelled cAMP was isolated and purified by chromatography on alumina and Dowex-50 resin and counted for ^{3}H and ^{14}C, as described previously (Haslam and McClenaghan, 1981; Haslam and Vanderwel, 1989). The [^{3}H]cAMP found in each sample was corrected for the recovery of [^{14}C]-cAMP and expressed as a percent of the total platelet ^{3}H.

2.6. Calculations

IC_{50} values for the inhibition by various compounds of the photolabelling of PDE III were defined as the concentrations causing a half-maximal inhibition. The maximum inhibition obtained varied from 80–100% in different experiments (see Results). Similarly, EC_{50} values for the stimulation of [^{3}H]cAMP accumulation were defined as the compound concentrations causing half-maximal increases in [^{3}H]cAMP. The latter were measured from a baseline corresponding to the [^{3}H]-cAMP level observed after incubation of platelets with 0.5 nM iloprost in the absence of the compound under study. Both IC_{50} and EC_{50} values (x_{50}) were determined using Fig. P (BIOSOFT, Ferguson, MO, USA) using the following equation:

$$y = \text{Min} + (\text{Max} - \text{Min})/\left(1 + (x/x_{50})^{-P}\right)$$

where y is the response (inhibition of photolabelling or increase in [^{3}H]cAMP), x the compound concentration, Min and Max the minimum and maximum values of y, and P the slope factor.

3. Results

3.1. Compounds that inhibit photolabelling of PDE III by [^{32}P]cGMP in human platelet cytosol

Photolabelling of platelet cytosol led to the marked incorporation of ^{32}P into platelet proteins of 110, 55, 49 and 38 kDa (Fig. 1). The 49 and 38 kDa proteins were usually resolved into doublets; the proportions of these subspecies varied in different experiments. We have previously shown that the 110 kDa labelled protein, which is present in platelet cytosol but not membranes, is the subunit of platelet PDE III, whereas the 49 and 55 kDa proteins probably represent the R_I and R_{II} subunits of cAMP-dependent protein kinase; the 38 kDa species may be proteolytic fragments of the latter proteins (Tang et al., 1993).

Photolabelling of PDE III was inhibited by cGMP or cAMP (Fig. 1, lanes 2 and 5) and by compounds known to decrease platelet PDE activity, such as trequinsin and cilostamide (lanes 8 and 9). Although 8-bromo-cGMP was also inhibitory (lane 3), its *Rp*-phosphorothioate derivative was inactive (lane 4). Similarly, neither *Sp*-cAMPS nor *Rp*-cAMPS had significant effects on the photolabelling of PDE III (lanes 6 and 7). Of the compounds tested in this experiment, only 20 μM cGMP inhibited ^{32}P incorporation into photolabelled species other than PDE III. Compounds

that inhibit Ca^{2+}-calmodulin-dependent phosphodiesterase (PDE I) (50 μM vinpocetine) or cAMP-specific phosphodiesterase (PDE IV) (100 μM rolipram) were without effect on the photolabelling of PDE III (not shown). Some labelling of an 80 kDa protein, previously identified as the partly soluble cGMP-dependent protein kinase (Tang et al., 1993), was observed in platelet cytosol, but only in the presence of 20 μM concentrations of cAMP, *S*p-cAMPS or PDE III inhibitors (Fig. 1).

The effects of increasing concentrations of unlabelled cGMP on the photolabelling of platelet cytosolic proteins were also studied (Fig. 2). The 110 kDa PDE III and certain weakly labelled 85–100 kDa and 61 kDa proteins, observed in some but not all experiments (compare Figs. 1 and 2), showed the highest affinity for cGMP, in that a marked (90%) inhibition of labelling was obtained at 100 nM (lane 3). The 85–100 kDa and 61 kDa species are likely to be proteolytic fragments of PDE III (Tang et al., 1993). The next most sensitive labelled protein was the lower component of the 49 kDa doublet, labelling of which was partly inhibited by 1 μM cGMP (lane 4). Photolabelling of other species was inhibited by higher cGMP concentrations (lane 5) (see also Fig. 1). Increased labelling of the 80 kDa protein was observed with 0.1–1 μM cGMP but not with higher concentrations of this cyclic nucleotide.

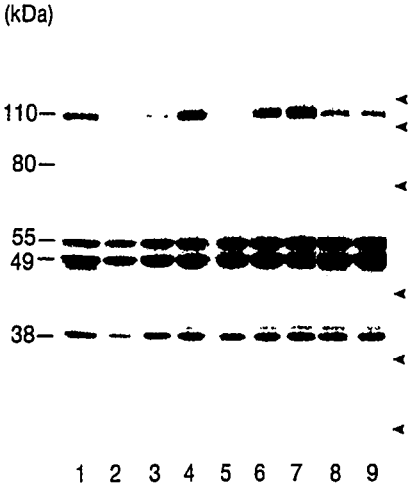

Fig. 1. Effect of various compounds on the photoaffinity labelling of human platelet supernatant proteins by [^{32}P]cGMP. Experimental conditions were as described under Materials and methods, with the addition of the following compounds: lane 1, none; lane 2, 20 μM cGMP; lane 3, 20 μM 8-bromo-cGMP; lane 4, 20 μM *R*p-8-bromo-cGMPS; lane 5, 20 μM cAMP; lane 6, 20 μM *S*p-cAMPS; lane 7, 20 μM *R*p-cAMPS; lane 8, 100 nM trequinsin; lane 9, 1 μM cilostamide. After irradiation, protein from each sample was analyzed by SDS-PAGE; an autoradiograph is shown. The positions of standard proteins are indicated on the right (see Materials) and the calculated masses of the labelled proteins are shown on the left.

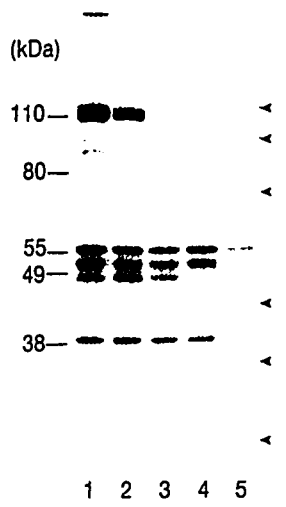

Fig. 2. Effects of unlabelled cGMP on the photoaffinity labelling of human platelet supernatant proteins by [^{32}P]cGMP. In this experiment, [^{32}P]cGMP was added last to incubation mixtures. Experimental additions were otherwise as described under Materials and methods, with the following additions: lane 1, none; lane 2, 10 nM cGMP; lane 3100 nM cGMP; lane 4, 1 μM cGMP; lane 5, 10 μM cGMP. After irradiation, protein from each sample was analyzed by SDS-PAGE; an autoradiograph is shown. The positions of standard proteins are indicated on the right and the calculated masses of the labelled proteins are shown on the left.

The effects of inhibitors of PDE III on the photolabelling of the enzyme were concentration-dependent, as shown for milrinone in Fig. 3. In this experiment, in which milrinone and the platelet protein were mixed before addition of [^{32}P]cGMP, the maximum inhibition of photolabelling approached 100%. Somewhat less inhibition (80–90%) was obtained when [^{32}P]cGMP was added to cytosol before PDE III inhibitors (e.g., Fig. 1). Inhibition of the labelling of the minor 85–100 kDa and 61 kDa proteins was also observed (e.g., Fig. 3), confirming that these species are likely to be proteolytic fragments of the 110 kDa PDE III. In the presence of concentrations of milrinone (or of other selective PDE III inhibitors) sufficient to block photolabelling of the 110 kDa species and its putative proteolytic fragments, a faint 105 kDa photolabelled protein often became visible (e.g., Fig. 3); this may represent the platelet cGMP-stimulated phosphodiesterase (PDE II). As noted previously for other PDE III inhibitors (Fig. 1), milrinone also induced a weak photolabelling of the 80 kDa protein.

Sufficient ^{32}P (700–1000 cpm) was incorporated into the 110 kDa protein present in 80 μg of cytosolic protein to permit quantitative measurements of the inhibitory effects on photolabelling of a range of concentrations of several PDE III inhibitors (Fig. 4). From a number of these experiments, each similar to that shown in Fig. 3, IC_{50} values (means ± S.E.M., $n = 3$ or

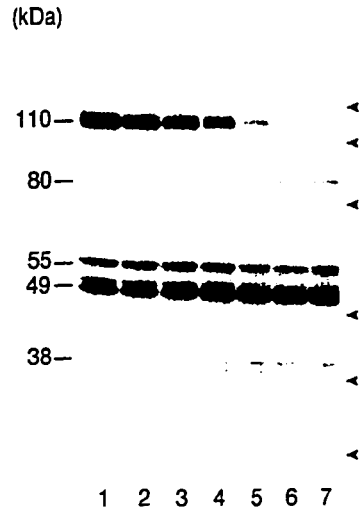

Fig. 3. Effect of increasing concentrations of milrinone on the photoaffinity labelling of human platelet PDE III by [^{32}P]cGMP. In this experiment, [^{32}P]cGMP was added last to incubation mixtures. Experimental conditions were otherwise as described under Materials and methods, with the addition of the following concentrations of milrinone: lane 1, none; lane 2, 20 nM; lane 3, 40 nM; lane 4, 100 nM; lane 5, 400 nM; lane 6, 1 μM; lane 7, 4 μM. After irradiation, protein from each sample was analyzed by SDS-PAGE; an autoradiograph is shown. The positions of standard proteins are indicated on the right and the calculated masses of labelled proteins including PDE III (110 kDa) on the left.

4) were calculated and were as follows: trequinsin 13 ± 2 nM; lixazinone, 22 ± 4 nM; milrinone 56 ± 12 nM; cilostamide 70 ± 9 nM; siguazodan, 117 ± 29 nM; IBMX, 3950 ± 22 nM. Provided IC_{50} values were defined as concentrations giving half-maximal inhibition, similar results were obtained whether or not [^{32}P]cGMP was added to incubation mixtures last. From the ^{32}P incorporated into PDE III and the enrichment of the enzyme during its purification to near homogeneity (Grant and Colman, 1984), we calculate that about 0.1% of the PDE III present in control samples was labelled after irradiation for 15 min.

3.2. Photoaffinity labelling of PDE III and other proteins in rat platelets and tissues

Photolabelling of rat platelet lysate with [^{32}P]cGMP (Fig. 5, lane 2) showed that the 110 kDa labelled protein found in human platelets was replaced by a labelled species that migrated significantly more slowly (at 115 kDa). This presumptive PDE III was present in much larger amounts in rat platelets than in other rat tissues (Fig. 5). However, it was detectable in almost all tissues studied and particularly in heart, uterus, spleen, liver, cerebrum and cerebellum. Rat platelet lysate and the rat tissues studied contained a major 49 kDa photolabelled protein that comigrated with that present in human platelet material. An 80 kDa labelled species, corresponding to that identified as cGMP-dependent protein kinase in human platelets (Tang et al., 1993), was detected in rat tissues, and most conspicuously in heart, aorta, uterus and lung (Fig. 5, lanes 3, 4, 5 and 8), but was labelled only weakly in rat platelet lysate (lane 2). The 38 kDa photolabelled proteins that were readily detected in human platelet material were virtually absent from rat platelets and skeletal muscle, though they were present in other rat tissues. A 175 kDa protein was labelled in many tissues, but most strongly in liver and spleen, and 35 and 28 kDa proteins were conspicuous in kidney homogenate.

Although most rat tissues contained much less of the 115 kDa labelled protein than rat platelets, it was

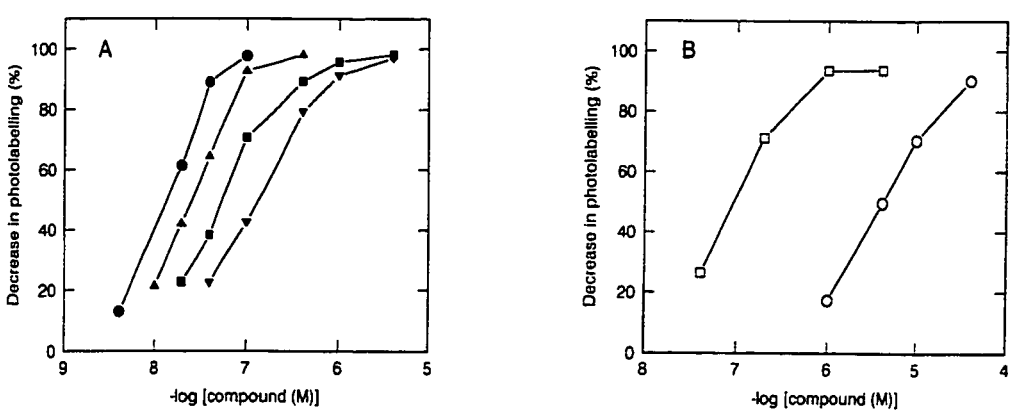

Fig. 4. Effects of various cAMP phosphodiesterase inhibitors on the photoaffinity labelling of human platelet PDE III by [^{32}P]cGMP. In these two experiments, [^{32}P]cGMP was added last to incubation mixtures. Platelet supernatant fraction was irradiated at 0°C in the presence of [^{32}P]cGMP and the indicated concentrations of the compounds listed below. Protein from the irradiated samples were analyzed by SDS-PAGE and the ^{32}P-labelled proteins detected by autoradiography. The 110 kDa labelled species (PDE III) was cut from the dried gels and counted for ^{32}P. Results with added compounds (means from 2 or 3 samples) are expressed as the percent decreases in labelling with respect to the control values (means of 3 or 4 samples). (A) ●, trequinsin; ▲, lixazinone; ■, milrinone; ▼, siguazodan. (B) □, cilostamide; ○, IBMX.

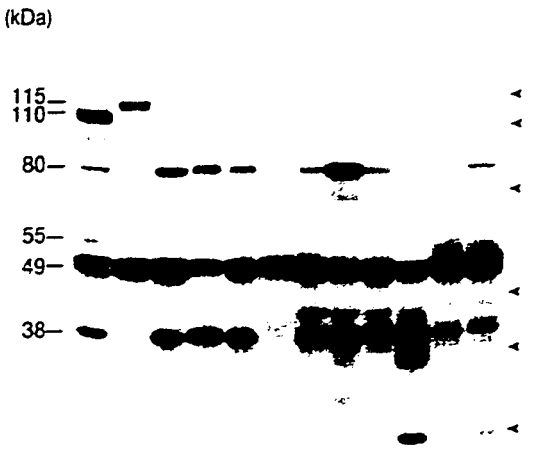

Fig. 5. Photoaffinity labelling of rat tissue homogenates by [^{32}P]cGMP. Samples containing 80 μg of protein from human or rat platelet lysate or rat tissue homogenate were irradiated as described under Materials and methods: lane 1, human platelets; lane 2, rat platelets; lane 3, rat heart; lane 4, rat aorta; lane 5, rat uterus; lane 6, rat skeletal muscle; lane 7, rat spleen; lane 8, rat lung; lane 9, rat liver; lane 10, rat kidney; lane 11, rat cerebrum; lane 12, rat cerebellum. After irradiation, protein from each sample was analyzed by SDS-PAGE; an autoradiograph is shown. The positions of standard proteins are indicated on the right and the calculated masses of the labelled proteins are shown on the left.

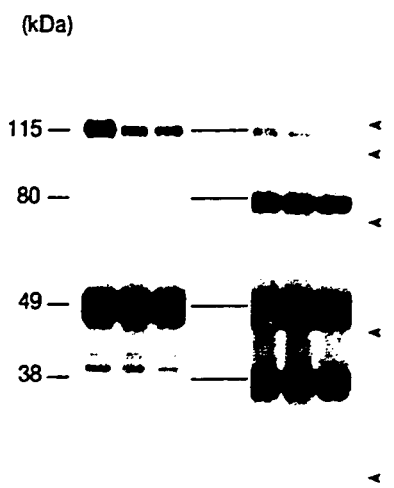

Fig. 6. Effects of PDE III inhibitors on the photoaffinity labelling of rat platelet lysate (lanes 1–3) and rat heart homogenate (lanes 4–6) by [^{32}P]cGMP. Experimental conditions were as described under Materials and methods, with the following additions: lanes 1 and 4, none; lanes 2 and 5, 1 μM cilostamide; lanes 3 and 6, 300 nM lixazinone. After irradiation, protein from each sample was analyzed by SDS-PAGE; an autoradiograph is shown. The positions of standard proteins are indicated on the right and the calculated masses of the labelled proteins are shown on the left.

possible to study the effects of competing compounds on the former after more prolonged exposure of autoradiographs, as shown for heart in Fig. 6. Thus, 1 μM cilostamide and 300 nM lixazinone each inhibited photolabelling of the 115 kDa protein to about the same extent in rat platelet lysate and rat heart homogenate without affecting the labelling of other proteins. This finding confirms that the 115 kDa protein is the rat PDE III.

3.3. Effects of PDE III inhibitors on cAMP accumulation in intact human platelets

To investigate the relationship between the abilities of the compounds studied to block photolabelling of PDE III and to increase cAMP in intact platelets, we measured changes in [^{3}H]cAMP in human platelets that had been prelabelled with [^{3}H]adenine. In 0.5 min incubations without other additions, trequinsin and cilostamide increased platelet [^{3}H]cAMP to about three-fold the basal level (Table 1). Since in absolute terms these increases in [^{3}H]cAMP were relatively small, detailed comparison of the effects of PDE inhibitors were made in the presence of 0.5 nM iloprost, a prostacyclin analogue that greatly enhances cAMP formation in platelets (Ashby, 1992). At this concentration, iloprost alone increased platelet [^{3}H]cAMP four- to seven-fold in different experiments (e.g., Table 1). Under these conditions, trequinsin, lixazinone, milrinone and siguazodan all increased the accumulation of [^{3}H]cAMP to similar maximum levels, which were two to four-fold the values obtained with iloprost alone

Table 1
Effects of PDE III inhibitors on the accumulation of cAMP in intact human platelets incubated without and with iloprost

PDE III inhibitor	[^{3}H]cAMP (% of platelet ^{3}H)	
	Without iloprost	With iloprost
None	0.027 ± 0.001	0.141 ± 0.011
Trequinsin (10 nM)	0.039 ± 0.001	0.297 ± 0.015
Trequinsin (100 nM)	0.061 ± 0.002	0.451 ± 0.036
Trequinsin (1 μM)	0.077 ± 0.002	0.523 ± 0.001
Cilostamide (100 nM)	0.034 ± 0.003	0.212 ± 0.006
Cilostamide (1 μM)	0.055 ± 0.001	0.366 ± 0.018
Cilostamide (10 μM)	0.080 ± 0.001	0.610 ± 0.007

Samples of washed platelets containing ^{3}H-labelled adenine nucleotides were incubated for 0.5 min at 37°C with the indicated concentrations of trequinsin or cilostamide in the absence or presence of 0.5 nM iloprost. [^{3}H]cAMP was isolated and expressed as a percent of the platelet ^{3}H; results are means ± S.E.M. from three identical samples.

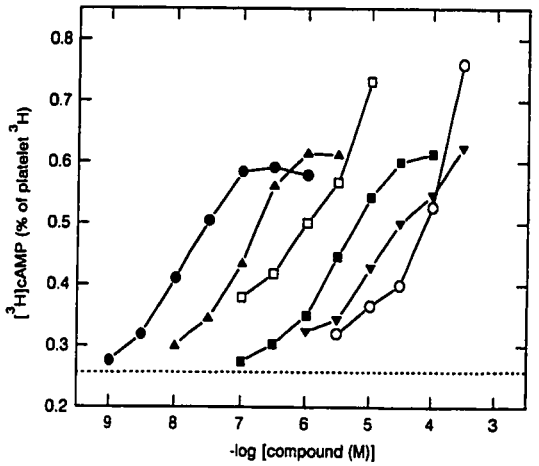

Fig. 7. Effects of various cAMP phosphodiesterase inhibitors on the accumulation of cAMP in intact human platelets. Samples of washed platelets containing ^{3}H-labelled adenine nucleotides were incubated for 0.5 min at 37°C in the presence of 0.5 nM iloprost and the indicated concentrations of the compounds listed below. [^{32}H]cAMP was isolated and expressed as a percent of the platelet ^{3}H. Results are means from three identical samples. The mean initial platelet [^{3}H]cAMP amounted to 0.04% of platelet ^{3}H and increased to 0.28% of platelet ^{3}H after 0.5 min incubation with iloprost (dashed line). The compounds studied were: ●, trequinsin; ▲, lixazinone; ■, milrinone; ▼, siguazodan; □, cilostamide; ○, IBMX.

(Table 1, Fig. 7). The most potent compound under the conditions of this assay was trequinsin (EC_{50}, 19 ± 3 nM); lixazinone, milrinone and siguazodan were about six-fold, 280-fold and 1000-fold less active, respectively (Fig. 7, Table 2). A different pattern of [^{3}H]cAMP accumulation was seen when either cilostamide or IBMX was used in the presence of iloprost, in that a larger accumulation of [^{3}H]cAMP was obtained and no maximum was observed within the concentration range studied (Table 1, Fig. 7). This suggests that high concentrations of the latter two compounds inhibited a PDE isoenzyme in addition to PDE III.

4. Discussion

We have previously shown that the 110 kDa protein photolabelled by [^{32}P]cGMP in human platelet cytosol is PDE III (Tang et al., 1993). This photolabelling of PDE III was inhibited by cGMP, 8-bromo-cGMP and cAMP, but not by phosphorothioate analogues of the latter two compounds, which bind to the cyclic nucleotide-dependent protein kinases (De Wit et al., 1984; Hofmann et al., 1985; Butt et al., 1990). The inhibition of photolabelling by low concentrations of unlabelled cGMP (≤ 100 nM) demonstrates the high affinity of the enzyme for cGMP. Published K_i values for the inhibition of PDE III from various sources by cGMP range from 35 to 60 nM (Grant and Colman, 1984; Bourne et al., 1984; Harrison et al., 1986), similar to the concentration of [^{32}P]cGMP selected for photolabelling (33 nM). Unlabelled cGMP, which inhibited photolabelling by 90% at 100 nM was thus more effective than expected. This may be accounted for by incomplete equilibration at 0°C of bound unlabelled cGMP with [^{32}P]cGMP supplied later, and is consistent with our observation of small differences in the maximum inhibition of photolabelling that depended on the order in which [^{32}P]cGMP and competing compounds were added. However, when experiments were carried out at room temperature to facilitate equilibration of PDE III with additions, photolabelling of the enzyme was greatly reduced, presumably as a result of the hydrolysis of [^{32}P]cGMP (unpublished results). We believe that the conditions used represent a satisfactory compromise between equilibration and degradation of the [^{32}P]cGMP.

Table 2
Comparison of the effects of PDE III inhibitors on the photolabelling of the human platelet enzyme by [^{32}P]cGMP with their abilities to increase cAMP accumulation in intact platelets and to inhibit purified platelet PDE III

Compound	IC_{50} for inhibition of photolabelling of PDE III (nM)	EC_{50} for stimulation of cAMP accumulation in platelets (nM)	Reported IC_{50} for inhibition of platelet PDE III (nM)
Trequinsin	13 ± 2 (3)	19 ± 3 (5)	0.25 [a]
Lixazinone	22 ± 4 (3)	122 ± 8 (5)	10 [c]
Milrinone	56 ± 12 (4)	5320 ± 970 (4)	460 [b], 860 [c]
Siguazodan	117 ± 29 (3)	18880 ± 3110 (3)	800 [d]
Cilostamide	70 ± 9 (3)	-	40 [b]
IBMX	3950 ± 22 (3)	-	2600 [b]

IC_{50} values for inhibition of the photolabelling of platelet PDE III (defined as the concentrations causing half-maximal inhibition) were determined in experiments similar to that shown in Fig. 3 and were calculated as described under Materials and methods. EC_{50} values for stimulation of cAMP accumulation in platelets were determined in the presence of 0.5 nM iloprost (see Fig. 7) and were calculated as the concentrations causing half-maximal increases in cAMP above the level seen with iloprost alone (see Materials and methods). These IC_{50} and EC_{50} values are means ± S.E.M. from the number of experiments shown in parentheses. Literature IC_{50} values for the inhibition of platelet PDE III, determined using partially purified enzyme and/or low (≤ 1 μM) cAMP concentrations, are also shown.
[a] Ruppert and Weithmann, 1982; [b] Macphee et al., 1986; [c] Venuti et al., 1988; [d] Murray et al., 1990.

Compounds described in the literature as potent inhibitors of platelet low K_m cAMP phosphodiesterase activity, including cilostamide (Hidaka et al., 1979), trequinsin (Ruppert and Weithmann, 1982), lixazinone (Alvarez et al., 1986), milrinone (Macphee et al., 1986) and siguazodan (Murray et al., 1990), were all found to inhibit the photolabelling of platelet PDE III by [^{32}P]-cGMP. With the exception of trequinsin, these compounds have been characterized as specific inhibitors of PDE III purified from platelets and/or cardiac muscle (Thompson, 1991). Although micromolar concentrations of trequinsin inhibit other phosphodiesterases and particularly PDE II (Souness et al., 1990; Whalin et al., 1991; Thompson, 1991), this compound is an exceptionally potent inhibitor of platelet phosphodiesterase activity (IC_{50}, 0.25 nM) under conditions in which only PDE III is active (Ruppert and Weithmann, 1982) and was the most effective inhibitor of PDE III photolabelling (Table 2). A non-specific inhibitor of PDE III, IBMX, also blocked photolabelling, but compounds that are specific inhibitors of other phosphodiesterases did not. As a whole, our results provide additional evidence that cAMP, cGMP and other PDE III inhibitors all bind to the same site, the active centre of the enzyme (Groppi et al., 1983; Grant and Colman, 1984; Beavo, 1988).

A comparison of the IC_{50} values of PDE III inhibitors for inhibition of photolabelling with published values for inhibition of enzyme activity is shown in Table 2. Similar results were obtained by these two methods when lixazinone, cilostamide or IBMX were studied, but trequinsin was more potent, and milrinone and siguazodan less potent in inhibiting enzyme activity than photolabelling. Differences in the purity (and proteolysis) of the platelet PDE III preparations and in the assays (particularly the cAMP concentrations) used by different authors may largely account for these discrepancies. As a whole, our results show that inhibition of photolabelling provides a simple and rapid method of identifying and comparing specific inhibitors of platelet PDE III.

Although the rank order of effectiveness of PDE III inhibitors in enhancing the cAMP accumulation induced by iloprost in intact human platelets was the same as for the inhibition of photolabelling of PDE III in cytosol, the quantitative discrepancies were large (Fig. 8, Table 2). Whereas trequinsin had essentially the same activity in these two assays, approximately 10-fold higher concentrations of lixazinone and 100-fold higher concentrations of milrinone or siguazodan were required to increase cAMP in platelets than to inhibit photolabelling. It is unlikely that these differences reflect an inhibition of PDE isoenzymes additional to PDE III, since the above four compounds increased platelet cAMP to the same maximum level. Differences in the rates of entry of these compounds into platelets may account for these results. Thus, for many PDE III inhibitors, much higher concentrations are required to inhibit platelet aggregation than to inhibit enzyme activity in broken cell preparations; this has been explained in terms of an optimum lipophilic window for permeation of platelets by these compounds (e.g., Meanwell et al., 1992). High concentrations of cilostamide or IBMX increased platelet cAMP above the maximum level obtained with the four compounds discussed so far. This strongly suggests that these two compounds inhibit an additional platelet cAMP phosphodiesterase, presumably PDE II which is found in small amounts in platelets (Grant et al., 1990). Cilostamide has, in fact, been reported to inhibit PDE II (Yamamoto et al., 1983). Our results therefore suggest that, despite its effects on PDE II (Thompson, 1991), trequinsin is a more specific inhibitor of PDE III than cilostamide. Purified bovine heart PDE II, which has a 105 kDa subunit, can be photolabelled by [^{32}P]-cGMP at two sites, one catalytic and the other regulatory (Stroop et al., 1989). A minor 105 kDa labelled species was detected in photolabelled platelet cytosol when labelling of PDE III and its putative proteolytic fragments were suppressed by milrinone.

We have previously shown that [^{32}P]cGMP photolabels cGMP-dependent protein kinase in platelet membranes and that addition of cAMP or *S*p-cAMPS greatly enhances this labelling (Tang et al., 1993). Small amounts of this 80 kDa protein were detected in platelet cytosol. We have now found that PDE III inhibitors also induce some labelling of this protein; it is possible that these compounds prevented degradation of traces of cAMP in platelet cytosol. Addition of a narrow range of concentrations of unlabelled cGMP also enhanced photolabelling of the 80 kDa protein, suggesting a cooperative interaction between cyclic nucleotide binding sites with different affinities for cGMP.

The results show that photolabelling with [^{32}P]cGMP can be used to detect differences in the amounts and molecular masses of cGMP-binding proteins in different tissues. Thus, in rat platelets and other rat tissues including heart, PDE III had a significantly larger subunit (115 kDa) than in human platelets. Although much more PDE III was photolabelled in rat platelets than in other rat tissues, we were able to demonstrate effects of PDE III inhibitors in heart homogenates. Photoaffinity labelling of PDE III therefore offers the possibility of studying tissue-specific actions of PDE III inhibitors without prior isolation of the enzyme from multiple sources. The most obvious differences between the rat tissues studied were in the amounts of the 80 kDa photolabelled protein, which by analogy with human platelets is likely to be cGMP-dependent protein kinase, and of the 38 kDa labelled proteins, which have been tentatively identified as proteolytic fragments of the regulatory subunits of cAMP-depend-

ent protein kinases (Tang et al., 1993). In contrast, the 49 kDa photolabelled species, which is probably the R_I subunit of cAMP-dependent protein kinase (Tang et al., 1993), was present in similar amounts in most rat tissues. Several unidentified minor photolabelled species also showed major tissue-specific differences. Although photoaffinity labelling with [^{32}P]cGMP is unlikely to provide an accurate measure of the relative amounts of these different cGMP-binding proteins in individual tissues because of variations in labelling efficiency, the distinctive labelling patterns that we have observed may provide useful indications of the predominant mechanisms of cyclic nucleotide action in different tissues, particularly as more is learned about the identities and functions of cGMP-binding proteins other than the classical cyclic nucleotide-dependent protein kinases.

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References

Alvarez, R., G.L. Banerjee, J.J. Bruno, G.L. Jones, K. Liittschwager, A.M. Strosberg and M.C. Venuti, 1986, A potent and selective inhibitor of cyclic AMP phosphodiesterase with potential cardiotonic and antithrombotic properties, Mol. Pharmacol. 29, 554.

Ashby, B., 1992, Comparison of iloprost, cicaprost and prostacyclin effects on cyclic AMP metabolism in intact platelets. Prostaglandins 43, 255.

Beavo, J.A., 1988, Multiple isozymes of cyclic nucleotide phosphodiesterase, in: Advances in Second Messenger and Phosphoprotein Research, vol. 22, ed. P. Greengard and G.A. Robison (Raven Press, New York) p. 1.

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.

Bourne, H.R., V.M. Brothers, H.R. Kaslow, V. Groppi, N. Walker and F. Steinberg, 1984, Genetic analysis of cyclic nucleotide phosphodiesterases in S49 mouse lymphoma cells, in: Advances in Cyclic Nucleotide and Protein Phosphorylation Research, vol. 16, ed. S.J. Strada and W.J. Thompson (Raven Press, New York) p. 185.

Butt, E., M. van Bemmelen, L. Fischer, U. Walter and B. Jastorff, 1990, Inhibition of cGMP-dependent protein kinase by (Rp)-guanosine 3',5'-monophosphorothioates, FEBS Lett. 263, 47.

De Wit, R.J.W., D. Hekstra, B. Jastorff, W.J. Stec, J. Baraniak, R. Van Driel and P.J.M. Van Haastert, 1984, Inhibitory action of certain cyclophosphate derivatives of cAMP on cAMP-dependent protein kinases, Eur. J. Biochem. 142, 255.

Erhardt, P.W., A.A. Hagedorn and M. Sabio, 1988, Cardiotonic agents. 3. A topographical model of the cardiac cAMP phosphodiesterase receptor, Mol. Pharmacol. 33, 1.

Grant, P.G. and R.W. Colman, 1984, Purification and characterization of a human platelet cyclic nucleotide phosphodiesterase, Biochemistry 23, 1801.

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, Thromb. Res. 59, 105.

Groppi, V.E., F. Steinberg, H.R. Kaslow, N. Walker and H.R. Bourne, 1983, Identification by direct photoaffinity labeling of an altered phosphodiesterase in a mutant S49 lymphoma cell, J. Biol. Chem. 258, 9717.

Harrison, S.A., D.H. Reifsnyder, B. Gallis, G.G. Cadd and J.A. Beavo, 1986, Isolation and characterization of bovine cardiac muscle cGMP-inhibited phosphodiesterase: a receptor for new cardiotonic drugs, Mol. Pharmacol. 29, 506.

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, in: Advances in Cyclic Nucleotide Research, vol. 9, ed. W.J. George and L.J. Ignarro (Raven Press, New York) p. 533.

Haslam, R.J. and J.A. Lynham, 1977, Relationship between phosphorylation of blood platelet proteins and secretion of platelet granule constituents I. Effects of different aggregating agents, Biochem. Biophys. Res. Commun. 77, 714.

Haslam, R.J. and M.D. McClenaghan, 1981, Measurement of circulating prostacyclin, Nature 292, 364.

Haslam, R.J. and M. Vanderwel, 1989, Measurement of changes in platelet cyclic AMP in vitro and in vivo by prelabeling techniques: application to the detection and assay of circulating PGI_2, in: Platelets: Receptors, Adhesion, Secretion, Part A, ed. J. Hawiger, Methods Enzymol. 169 (Academic Press, New York) p. 455.

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.

Hofmann, F., H.-P. Gensheimer, W. Landgraf, R. Hullin and B. Jastorff, 1985, Diastereomers of adenosine 3',5'-monothionophosphate (cAMP[S]) antagonize the activation of cGMP-dependent protein kinase, Eur. J. Biochem. 150, 85.

Laemmli, U.K., 1970, Cleavage of structural proteins during the assembly of the head of bacteriophage T4, Nature 227, 680.

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.

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. USA 83, 6660.

Maurice, D.H. and R.J. Haslam, 1990, 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.

Meanwell, N.A., B.C. Pearce, H.R. Roth, E.C.R. Smith, D.L. Wedding, J.J.K. Wright, J.O. Buchanan, U.M. Baryla, M. Gamberdella, E. Gillespie, D.C. Hayes, S.M. Seiler, H.C. Stanton, G.B. Zavoico and J.S. Fleming, 1992, Inhibitors of blood platelet cAMP phosphodiesterase. 2. Structure-activity relationships associated with 1,3-dihydro-2*H*-imidazo[4,5-*b*]quinolin-2-ones substituted with functionalized side chains, J. Med. Chem. 35, 2672.

Murray, K.J. and P.J. England, 1992, Inhibitors of cyclic nucleotide phosphodiesterases as therapeutic agents, Biochem. Soc. Trans. 20, 460.

Murray, K.J., P.J. England, T.J. Hallam, J. 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.

Mustard, J.F., D.W. Perry, N.G. Ardlie and M.A. Packham, 1972, Preparation of suspensions of washed platelets from humans, Br. J. Haematol. 22, 193.

Reeves, M.L., B.K. Leigh and P.J. England, 1987, The identification of a new cyclic nucleotide phosphodiesterase activity in human and guinea-pig cardiac ventricle. Implications for the mechanism of action of selective phosphodiesterase inhibitors, Biochem. J. 241, 535.

Reifsnyder, D.H., S.A. Harrison, C.H. Macphee, M.B. McCormack and J.A. Beavo, 1985, Antibody and inhibitor analysis of cGMP-inhibited phosphodiesterase in mutant S49 cells and rat adipocytes, Fed. Proc. 44, 1816.

Ruppert, D. and K.U. Weithmann, 1982, HL 725, an extremely potent inhibitor of platelet phosphodiesterase and induced platelet aggregation in vitro, Life Sci. 31, 2037.

Saitoh, S., T. Saito, A. Otake, T. Owada, M. Mitsugi, H. Hashimoto and Y. Maruyama, 1993, Cilostazol, a novel cyclic AMP phosphodiesterase inhibitor, prevents reocclusion after coronary arterial thrombolysis with recombinant tissue-type plasminogen activator, Arterioscler. Thromb. 13, 563.

Seiler, S., E. Gillespie, A.J. Arnold, C.L. Brassard, N.A. Meanwell and J.S. Fleming, 1991, Imidazoquinoline derivatives: potent inhibitors of platelet cAMP phosphodiesterase which elevate cAMP levels and activate protein kinase in platelets, Thromb. Res. 62, 31.

Souness, J.E., B.K. Diocee, W. Martin and S.A. Moodie, 1990, Pig aortic endothelial-cell cyclic nucleotide phosphodiesterases. Use of phosphodiesterase inhibitors to evaluate their roles in regulating cyclic nucleotide levels in intact cells, Biochem. J. 266, 127.

Stroop, S.D., H. Charbonneau and J.A. Beavo, 1989, Direct photolabeling of the cGMP-stimulated cyclic nucleotide phosphodiesterase, J. Biol. Chem. 264, 13718.

Tang, K.M., J.L. Sherwood and R.J. Haslam, 1993, Photoaffinity labelling of cyclic GMP-binding proteins in human platelets, Biochem. J. 294, 329.

Thompson, W.J., 1991, Cyclic nucleotide phosphodiesterases: pharmacology, biochemistry and function, Pharmacol. Ther. 51, 13.

Venuti, M.C., R.A. Stephenson, R. Alvarez, J.J. Bruno and A.M. Strosberg, 1988, Inhibitors of cyclic AMP phosphodiesterase. 3. Synthesis and biological evaluation of pyrido and imidazolyl analogues of 1,2,3,5-tetrahydro-2-oxoimidazo[2,1-*b*]quinazoline, J. Med. Chem. 31, 2136.

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, Biochem. Pharmacol. 35, 787.

Whalin, M.E., J.G. Scammell, S.J. Strada and W.J. Thompson, 1991, Phosphodiesterase II, the cGMP-activatable cyclic nucleotide phosphodiesterase, regulates cyclic AMP metabolism in PC12 cells, Mol. Pharmacol. 39, 711.

Yamamoto, T., S. Yamamoto, J.C. Osborne, V.C. Manganiello and M. Vaughan, 1983, Complex effects of inhibitors on cyclic GMP-stimulated cyclic nucleotide phosphodiesterase, J. Biol. Chem. 258, 14173.

CHAPTER 4

PUBLISHED MANUSCRIPT #3

Tang, K. Mary, Elliott K. Jang and Richard J. Haslam. (1996) Expression and mutagenesis of the catalytic domain of cGMP-inhibited phosphodiesterase (PDE3) cloned from human platelets. *Biochem. J.* (in press).

PREAMBLE

The purpose of these studies was to investigate the molecular mechanisms by which cGMP and inhibitory drugs regulate PDE3 activity. Molecular cloning, mutagenesis and expression studies were used to characterize the roles of different domains of PDE3 in the actions of cGMP and selected compounds.

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Expression and mutagenesis of the catalytic domain of cGMP-inhibited phosphodiesterase (PDE3) cloned from human platelets

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ABSTRACT

We have used reverse transcriptase-PCR, platelet mRNA and degenerate primers based on platelet peptide sequences, to amplify a fragment of platelet cGMP-inhibited phosphodiesterase (cGI-PDE; PDE3). Sequence analysis of this clone established that both the platelet and cardiac forms of PDE3 were derived from the same gene (PDE3A). A RT-PCR product representing the C-terminal half of platelet PDE3 cDNA and corresponding to amino acid residues 560-1141 of the cardiac enzyme, was cloned and expressed in *Escherichia coli* (cGI-PDEΔ1). Further deletion mutants were constructed by removing either an additional 100 amino acids from the N-terminus (cGI-PDEΔ2) or the 44 amino acid insert, characteristic of the PDE3 family, from the catalytic domain (cGI-PDEΔ1Δi). In addition, site-directed mutagenesis was performed to explore the function of the 44 amino acid insert. All mutants were evaluated for their ability to hydrolyse cAMP and cGMP, their ability to be photolabelled by [^{32}P]cGMP and for the effects of PDE3 inhibitors. The K_m values for hydrolysis of cAMP and cGMP by immunoprecipitates of cGI-PDEΔ1 (182 ± 12 nM and 153 ± 12 nM, respectively) and cGI-PDEΔ2 (131 ± 17 nM and 99 ± 1 nM, respectively) were significantly lower than those for immunoprecipitates of intact platelet PDE3 (398 ± 50 nM and 252 ± 16 nM, respectively). Moreover, N-terminal truncations of platelet enzyme increased the ratio of V_{max} for cGMP/V_{max} for cAMP from 0.16 ± 0.01 in intact platelet enzyme, to 0.37 ± 0.05 in cGI-PDEΔ1 and to 0.49 ± 0.04 in cGI-PDEΔ2. Thus, deletion of the N-terminus enhanced hydrolysis of cGMP relative to cAMP, suggesting that N-terminal sequences may exert selective effects on enzyme activity. Removal of the 44 amino acid insert generated a mutant

with a catalytic domain closely resembling those of other PDE gene families but, despite a limited ability to be photolabelled by [^{32}P]cGMP, no cyclic nucleotide hydrolytic activities of the mutant were detectable. Mutation of amino acid residues in putative β-turns at the beginning and end of the 44 amino acid insert to alanine residues markedly reduced the ability of the enzyme to hydrolyse cyclic nucleotides. The PDE3 inhibitor, lixazinone, retained the ability to inhibit cAMP hydrolysis and [^{32}P]cGMP binding by the N-terminal deletion mutants and the site-directed mutants, suggesting that PDE3 inhibitors may interact exclusively with the catalytic domain of the enzyme.

Abbreviations used: PDE, phosphodiesterase; cGI-PDE, cGMP-inhibited phosphodiesterase; TBS, Tris-buffered saline; RT, reverse transcriptase; HEL, human erythroleukemia; EHNA, *erythro*-9-(2-hydroxy-3-nonyl)adenine.

INTRODUCTION

In human platelets, increases in cAMP or cGMP inhibit agonist-induced platelet aggregation [1-4]. Intracellular concentrations of cyclic nucleotides are tightly controlled by the activities of adenylyl and guanylyl cyclases and various phosphodiesterases (PDEs). Three distinct forms of PDEs were initially isolated by DEAE-cellulose chromatography of platelet extracts [5]. Further studies in platelets led to the identification and purification of a low K_m-cAMP phosphodiesterase that was competitively inhibited by cGMP [6,7], a cGMP-binding cGMP phosphodiesterase [8,9] and a cGMP-stimulated phosphodiesterase that hydrolyses both cAMP and cGMP [10]. Seven mammalian PDE gene families have now been recognized and classified according to substrate affinities, inhibitor sensitivities, modes of regulation and primary sequence homology, and a standardized nomenclature for these enzymes has now been adopted [11]. According to this classification, platelet cGMP-inhibited phosphodiesterase (cGI-PDE), cGMP-stimulated phosphodiesterase (cGS-PDE) and cGMP-binding phosphodiesterase (cGB-PDE) are members of the PDE3, PDE2 and PDE5 gene families, respectively. Hydrolysis of cGMP results predominantly from PDE 5, an enzyme possessing a high K_m for cAMP and a low K_m for cGMP. Although PDE2 and PDE3 enzymes both hydrolyse cAMP, they are differentially regulated by cGMP. PDE2, for instance, is stimulated by cGMP through an allosteric binding site, whereas PDE3 is competitively inhibited by cGMP, which is hydrolysed very slowly. In platelets, the PDE3 enzyme is the major cAMP phosphodiesterase and is thought to account for 80% of the hydrolysis at submicromolar concentrations of cAMP [12]. Increases in platelet cAMP attributed to the inhibition of PDE3 by

cGMP, have been shown to account for the synergistic inhibition of platelet aggregation by activators of guanylyl and adenylyl cyclases [13].

All PDEs contain a conserved 270 amino acid segment located in the carboxyl terminus of the molecule which encompasses the catalytic domain of the enzyme [14,15]. The highly divergent amino terminal regions of the PDE molecules are likely to confer some of the distinctive regulatory properties characteristic of the individual PDE gene families. cDNAs encoding two distinct cGMP-inhibited PDEs have been previously cloned from a human cardiac cDNA library (PDE3A) [16] and a rat adipocyte cDNA library (PDE3B) [17]. Both PDE3A and PDE3B possess a common domain organization [18]. The deduced amino acid sequences of these two subfamily members indicate a high degree of homology (88%) at the C-terminal catalytic region and a significant amount of divergence at the N-terminus. Located within the catalytic domain is a 44 amino acid insert which is characteristic of the PDE3 gene family. Although the function of this insert is not known, it could play a role in substrate binding or interaction with PDE3-specific inhibitors. In this study, we have used reverse-transcriptase PCR (RT-PCR) to clone the C-terminal half of human platelet cGI-PDE. The resulting truncation mutant, cGI-PDEΔ1 was expressed in *Escherichia coli*, purified for kinetic and pharmacologic analysis and compared with the intact platelet enzyme. The effects of deletion and site-directed mutagenesis of the 44 amino acid insert were also studied.

EXPERIMENTAL

Materials

[^{3}H]cAMP (27 Ci/mmole), [^{3}H]cGMP (9.3 Ci/mmole), [^{14}C]AMP (590 mCi/mmole) and [^{14}C]GMP (50 mCi/mmole) were obtained from Du Pont (Mississauga, ON, Canada). [^{32}P]cGMP (3000 Ci/mmole) was supplied by ICN Pharmaceuticals Inc. (Costa Mesa, CA, U.S.A). Leupeptin, heparin, Protein A-Sepharose, cAMP, cGMP, Nonidet P40, PMSF and protein standards (myosin heavy chain, 205 kDa; β-galactosidase, 116 kDa; phosphorylase *b*, 97.4 kDa; BSA, 66 kDa; ovalbumin, 45 kDa; glyceraldehyde-3-phosphate dehydrogenase, 36 kDa; carbonic anhydrase, 29 kDa) were purchased from Sigma (St. Louis, MO, U.S.A.). Immobilon P was obtained from Millipore (Canada) Ltd. (Mississauga, ON, Canada), *Taq* polymerase from Gibco/BRL (Burlington, ON, Canada) and all restriction enzymes from New England BioLabs (Beverly, MA, U.S.A.). The following compounds were gifts: zaprinast (May & Baker, Dagenham, Essex, U.K.), lixazinone (Syntex Corporation, Palo Alto, CA, U.S.A.), milrinone (Sterling Drug, Rensselaer, NY, U.S.A.) and rolipram (Schering, Berlin, Germany). *Erythro*-9-(2-hydroxy-3-nonyl)adenine (EHNA) was generously provided by Dr. T. Podzuweit (Max Planck Institute, Bad Nauheim, Germany). All oligonucleotide primers were synthesized by the Institute for Molecular Biology and Biotechnology (MOBIX) at McMaster University.

Preparation of platelet soluble proteins

Human platelets were first isolated and washed in Ca^{2+}-free Tyrode's solution supplemented with 5 mM Pipes, pH 6.5, 50 units of heparin/ml, 75 μg of apyrase/ml

and 3.5 mg of BSA/ml) as described by Mustard *et al.* [19]. After centrifugation at 1000 g for 15 min, the platelets were finally resuspended at 2.5 x 10^9/ml in Buffer A (100 mM KCl, 2.5 mM EGTA, 25 mM Hepes, pH 7.4, and 0.2 mM leupeptin), frozen in 1 ml aliquots using a solid CO_2/methanol bath and stored at -70 °C. When required, the platelets were thawed and sonicated on ice for 4 x 30 s; soluble proteins were obtained by centrifugation at 100,000 g for 1 h. These proteins were used either for photolabelling or for immunoprecipitation (see below).

Reverse transcriptase PCR

Human platelet mRNA was isolated using the FastTrack mRNA Isolation kit (Invitrogen, San Diego, CA, U.S.A.) according to the manufacturer's instructions. Platelet cDNA was then prepared using Superscript reverse transcriptase (Gibco/BRL, Burlington, ON, Canada) and an oligo (dT) primer (1 h at 37 °C), after which the sample was heated to 99 °C for 5 min to inactivate the enzyme. Degenerate primers, each with two 5′ restriction sites (in bold) were designed based on the sequences of peptides isolated from platelet cGI-PDE, and also found in the cloned human cardiac enzyme [16] (forward primer: 5′-GG**TCTAGAGGATCC**(C/A/T/G)GA(G/A)AC (A/T/C/G)ATGATGTT-3′, corresponding to amino acid residues 661-666 of the cardiac PDE3; reverse primer: 5′GG**GAATTCGTCGAC**(G/A)AA(G/A)TC(G/A) AA(G/A)TG(C/T)TT-3′, corresponding to amino acid residues 911-917). An 800 bp PCR product generated using these primers and platelet cDNA as a template was purified by low-melting point agarose gel electrophoresis (FMC BioProducts, Rockland, ME, U.S.A.), cloned into pBluescript II SK^+ (Stratagene, La Jolla, CA,

U.S.A) and sequenced in both directions using Sequenase Version 2 (Amersham, Oakville, ON, Canada). PCR reaction conditions using *Taq* DNA polymerase were: first 5 cycles, denaturation for 1 min at 95 °C, annealing for 2 min at 45 °C and extension for 1.5 min at 65 °C, followed by 25 cycles of 1 min at 95 °C, 2 min at 65 °C, and 1.5 min at 72 °C, with a final extension step of 10 min at 72 °C.

Following demonstration that this PCR fragment was identical in sequence with human cardiac cGI-PDE (PDE3A) cDNA, sequence-specific forward and reverse primers were selected to amplify the C-terminal half of the platelet cDNA, including the whole of the catalytic domain (Figure 1, **a**). This truncation mutant (cGI-PDEΔ1) was generated with primers 1 and 2, constructed with *Bam*HI and *Eco*RI restriction sites, respectively (forward: 5′-TA**GGATCC**CACAGGGCCTTAACTTACACTCAG-3′ and reverse: 5′-TC**GAATTC**GGTACCATTGTCACTGGTCTGGCT-3′), yielding sequence corresponding to amino acids 560-1141 of the cardiac enzyme. In addition, a shorter truncation mutant (cGI-PDEΔ2) was generated using the forward degenerate primer to yield sequence corresponding to amino acid residues 659-1141.

Removal of the 44 amino acid insert characteristic of the PDE3 family (shown in black) from the catalytic domain of cGI-PDEΔ1 was achieved using a PCR approach (Figure 1, **b**). Regions upstream and downstream of this insert were selectively amplified, purified on a low-melting point agarose gel, and ligated to generate a mutant, cGI-PDEΔ1Δi. To conserve the amino acid sequence, PILS, at the site of ligation, *Afl*II restriction sites were incorporated into PCR primers 3 and 4 (Figure 1,

b). For construction of the above deletion mutants, the PCR profile consisted of 25 cycles containing denaturation at 95 °C for 1 min, primer annealing at 58 °C for 2 min and chain extension at 65 °C for 1.5 min. The extension step of the final cycle was increased to 10 min at 72 °C. All PCR products were purified, cloned and sequenced in pBluescript II SK^+, as above.

Expression and purification of fusion proteins

cGI-PDE deletion mutants were subcloned into the *Bam*HI and *Kpn*I restriction sites of the bacterial expression vector pQE-30 (Qiagen, Chatsworth, CA, U.S.A.) to yield fusion proteins with an N-terminal $MRGSH_6GS$ sequence. The *E.coli* M15 strain transformed with this plasmid were grown to an optical density of between 0.5 - 0.6 before being induced with 0.5 mM isopropyl thiogalactopyranoside. After growth at 30 °C for an additional 4 h, the cells were harvested and resuspended either in Buffer B (50 mM NaH_2PO_4, pH 7.8, 300 mM NaCl, 1 mM PMSF, 0.2 mM leupeptin) for isolation of His_6-tagged protein by nickel-resin chromatography, or in Buffer C (100 mM Hepes, pH 7.5, 2 mM EGTA, 0.2 mM leupeptin) for immunoprecipitation of protein. In both cases, bacterial suspension was sonicated on ice for 4 x 30 s and centrifuged at 100,000 g for 1 h. Supernatant proteins in Buffer B were loaded onto a nickel-resin column to bind the histidine-containing fusion protein. Contaminating proteins were removed by washing with Buffer B containing increasing concentrations of imidazole (5, 80, and 100 mM). Intact His_6-tagged protein was then eluted using Buffer B containing 500 mM imidazole. The eluant was dialysed overnight against Buffer C to remove imidazole prior to enzymatic analysis. Alternatively, the expressed

protein was immunoprecipitated from bacterial supernatant proteins in Buffer C using anti-PDE3A IgG, affinity-purified on Avid gel (BioProbe Inc., Tustin, CA, U.S.A.). Protein samples (100 µl) were incubated for 90 min with 20-30 µl of IgG at 0°C, and were then shaken for 90 min at 4°C with 50 µl of 50% Protein A Sepharose. The Protein A Sepharose was isolated by centrifugation (12,000 g, 2 min), washed three times with Buffer C, and resuspended in an appropriate volume of Buffer C for assay of cAMP and cGMP phosphodiesterase activities.

Site-directed mutagenesis of cGI-PDEΔ1

Mutagenesis was performed according the 2-step PCR protocol of Landt *et al.*[20] with minor modifications. Briefly, an intermediate PCR product was first generated using a 5′ sequence-specific primer and a 3′ mutagenic primer containing the modified deoxynucleotides. This fragment was purified on a low-melting point agarose gel and used as the 5′ mutagenic primer in a second PCR reaction with a 3′ sequence-specific primer. The sequence-specific primers were 5′ and 3′ to unique restriction sites present in cGI-PDEΔ1. The PCR products were digested with appropriate restriction enzymes and subcloned into the corresponding regions of the pBluescript II SK^+ containing cGI-PDEΔ1. These mutant clones were sequenced and then further subcloned into pQE-30 for expression.

Western blotting

Bacterial proteins were separated by SDS/PAGE using 10 % acrylamide and transferred electrophoretically (120 mA for 3 h) onto Immobilon P membrane. A

solution of 5 % (w/v) dried milk in Tris-buffered saline (TBS: 10 mM Tris-HCl, pH 8.0, 150 mM NaCl) was used to block the membrane. The primary anti-PDE3A antibody [21] was diluted 1:500 in TBS and incubated with membranes at room temperature for 1 h. Membranes were washed three times with TBS containing 0.05 % (v/v) Nonidet-P40 and once with TBS, before incubation with secondary antibody conjugated with alkaline phosphatase and detection of immune complexes (Promega, Unionville, ON, Canada).

Measurement of PDE activity

PDE assays (0.1 ml) were performed in the presence of 50 mM Hepes, pH 7.5/1.0 mM EGTA/5 mM $MgCl_2$/0.1 mM leupeptin. Nickel-purified or immunoprecipitated enzyme was incubated with [^{3}H]cAMP or [^{3}H]cGMP for 5 or 10 min at 30 °C. Reactions were terminated by adding 0.1 ml of 10 mM EDTA and boiling for 2 min. Approximately 2000 d.p.m. of [^{14}C]AMP or 4000 d.p.m. of [^{14}C]GMP was added to each sample to monitor recovery of [^{3}H]AMP or [^{3}H]GMP which were isolated on Affi-Gel 601 Boronate affinity columns (Bio-Rad, Mississauga, ON, Canada) by a modification of the method of Davis and Daly [22]. Each column (0.8 ml bed volume) was washed with 16 ml of buffer containing 50 mM Hepes, pH 8.5 and 100 mM $MgCl_2$. [^{3}H]AMP or [^{3}H]GMP was then eluted with 2 ml of 250 mM acetic acid and counted for ^{3}H and ^{14}C in 10 ml of ACS scintillation fluid, using a Beckman LS5000TA counter. All assays were performed in triplicate or quadruplicate. Contaminating [^{3}H]AMP or [^{3}H]GMP were removed from the tritiated substrates ([^{3}H]cAMP and [^{3}H]cGMP) before their use in the assay by passing solutions of these

substrates through Affi-Gel 601 Boronate columns. All IC_{50} values and enzyme kinetic data (K_m and V_{max}) were determined using Grafit version 3.0 (Erithacus Software Ltd., Staines, U.K.).

Photoaffinity labelling of proteins by [^{32}P]cGMP

The method used has been described previously [21]. Briefly, incubation mixtures (0.1 ml) containing 80-100 μg of total bacterial supernatant protein in Buffer A and 5 μCi of [^{32}P]cGMP (33 nM after dilution) were stirred at 0 °C for 15 min in the dark and then for a further 15 min under a UV light. The proteins were precipitated with trichloroacetic acid and analyzed by SDS/PAGE (10 % acrylamide). Autoradiography was performed using Kodak X-OMAT AR film.

RESULTS

Cloning, bacterial expression and purification of the human platelet cGI-PDE

Degenerate primers, based upon platelet peptide sequences, and purified platelet mRNA were used in an RT-PCR reaction to determine whether the platelet PDE3 enzyme was distinct from the previously cloned human cardiac enzyme [16]. An 800 bp PCR product was generated and cloned into pBluescript II SK$^+$. Nine independent clones were sequenced in both directions, and shown to be identical to nucleotide residues 1982-2752 of the cardiac enzyme (PDE3A). This established that both the platelet and cardiac forms of PDE3 are derived from the same gene. However, this result does not exclude the possibility of alternate splicing of the platelet enzyme at the N-terminus.

Sequence-specific primers were selected for RT-PCR amplification of the C-terminal half of the platelet enzyme including the whole of the catalytic domain (Figure 1**a**). The 1769 bp PCR product (see Figure 1**a**, photograph insert), which represents amino acids 560-1141 of the cardiac enzyme, was cloned into the pBluescript II SK^+ vector for sequencing. A *Bam*HI/*Kpn*I cDNA fragment was inserted into the corresponding restriction sites of the bacterial expression vector pQE-30. A His_6-tagged protein representing a truncated form of platelet cGI-PDE (cGI-PDEΔ1) was expressed to high levels in *E. coli*. A further deletion mutant, cGI-PDEΔ2, containing amino acid residues 659-1141, was constructed by replacing the forward PCR primer (see Experimental section). In addition, the deletion of the 44 amino acid insert from the catalytic domain was achieved by selective PCR amplification of upstream and downstream regions (cGI-PDEΔ1Δi). Amino acid residues surrounding the insert (-PI[insert]LS-) were conserved by incorporating an *Afl*II restriction site into primers 3 and 4 (Figure 1**b**).

The His_6-tagged proteins were purified from bacterial supernatants using nickel-resin column chromatography (Figure 2**a**) or were selectively immunoprecipitated using anti-PDE3A antibody that binds to the C-terminal 14 amino acids of the enzyme [21]. An immunoblot of the immunoprecipitates (Figure 2**b**) indicated that the apparent molecular weights of the cGI-PDEΔ1, cGI-PDEΔ1Δi and cGI-PDEΔ2 mutants were higher (80, 75 and 66 kDa respectively) than their calculated molecular weights (67, 62 and 56 kDa respectively). The decrease in electrophoretic mobility of these fusion proteins probably results from the presence of the N-terminal His_6 tag.

Photoaffinity labelling of bacterial extracts expressing platelet cGI-PDE deletion mutants

The cGI-PDEΔ1 and cGI-PDEΔ2 mutants were readily photolabelled by [^{32}P]cGMP in proportion to the amounts of expressed protein present (compare Figure 2**b**, lanes 1 and 3, with Figure 3, lanes 2 and 4). Despite the fact that the cGI-PDEΔ1Δi mutant protein was expressed to the same level as cGI-PDEΔ1, photolabelling of the former by [^{32}P]cGMP was much weaker than the latter (Figure 3, lane 3). All three photolabelled species were absent from bacterial lysate transformed with the control pQE-30 vector alone (Figure 3, lane 1). These results indicate that although the expressed N-terminal deletion mutants retained the ability to bind cGMP, removal of the 44 amino acid insert from the catalytic domain greatly reduced cGMP binding. Thus, cGMP binds to the 62 kDa C-terminal part of PDE3 and, for structural or other reasons, the 44 amino acid insert facilitates this interaction.

The effects of PDE3 inhibitors on the photolabelling of cGI-PDEΔ1 and cGI-PDEΔ2 were studied. Lixazinone, milrinone and cGMP, all of which inhibit the activity of intact cGI-PDE, suppressed photolabelling of these deletion mutants by [^{32}P]cGMP (Figure 3**b**). These results confirm that all three compounds interact with the C-terminal part of the enzyme containing the catalytic domain.

Comparison of the enzymic properties of cGI-PDE deletion mutants with that of the intact platelet enzyme

Nickel-resin purified cGI-PDEΔ1 was assayed for enzyme activity. Kinetic analysis

indicated that the K_m values for cAMP and cGMP hydrolysis were 153 nM and 175 nM respectively, and that the ratio of V_{max} for cGMP/V_{max} for cAMP was 0.36. On the basis of the ratios of V_{max}/K_m, the inhibition of cGI-PDEΔ1 by lixazinone was mixed, with approximately equal competitive and uncompetitive components. Although good kinetic plots were obtained, the enzyme activity of the nickel-resin purified preparation was unstable and was lost within 2-3 days when the enzyme was stored at 4 °C. The sensitivity of the nickel-resin to strong reducing agents, chelating agents and ionic detergents restricted the steps that could be taken to stabilize the enzyme during purification, and dialysis to remove the high concentration of imidazole used to elute the enzyme, may have increased the likelihood of inactivation. For these reasons, this method was not routinely used and measurement of enzyme activity in immunoprecipitates was adopted for comparison of the expressed mutant enzymes with the platelet enzyme. The immunoprecipitation procedure provided a more rapid means of enzyme purification that minimized the risk of proteolysis.

The kinetic properties of the immunoprecipitated cGI-PDEΔ1 mutant were very similar to those of the nickel-resin purified enzyme. The K_m values for hydrolysis of cAMP and cGMP were 182 ± 12 nM and 153 ± 12 nM respectively, whereas, the ratio of V_{max} for cGMP/V_{max} for cAMP was 0.37 ± 0.05 (Table 1). Since the latter value was very different from published results with the intact cGI-PDE [23], we directly compared the K_m values and V_{max} ratios of our expressed truncation mutants with that of the immunoprecipitated intact platelet enzyme (Table 1). A trend towards progressively lower K_m values accompanied the deletion of N-terminal sequences from

cGI-PDE. Although both cGI-PDEΔ1 and cGI-PDEΔ2 showed significantly lower K_m values for cAMP and cGMP than intact enzyme, only the K_m of cGI-PDEΔ2 for cGMP was significantly lower than that of cGI-PDEΔ1 ($P<0.05$, Student t test). Moreover, successive deletions of the N-terminus led to an increase in the ratio V_{max} for cGMP/V_{max} for cAMP from 0.16 ± 0.01 in intact cGI-PDE, to 0.37 ± 0.05 in cGI-PDEΔ1 and to 0.49 ± 0.04 in cGI-PDEΔ2. Thus, our results indicate that loss of the N-terminus enhanced the hydrolysis of cGMP relative to that of cAMP, implying that N-terminal sequences may exert a selective effect on the activity of the catalytic domain.

The effects of PDE3 inhibitors on the immunoprecipitated enzymes were studied. Lixazinone showed the same pattern of mixed inhibition of immunoprecipitated cGI-PDEΔ1 as it did with the nickel-resin purified enzyme (not shown). However, in the case of the immunoprecipitated platelet enzyme, inhibition by lixazinone could be characterized as competitive or predominantly competitive. This kinetic profile was distinct from that of the expressed cGI-PDEΔ1 . Measurements of IC_{50} values for inhibition of cAMP hydrolysis by lixazinone and milrinone (Table 1) indicated three-fold increases for the deletion mutants cGI-PDEΔ1 and cGI-PDEΔ2, relative to the intact platelet enzyme. This must largely reflect decreases in the K_m for cAMP rather than reduced affinities for the inhibitors. Comparison of the effects of selective inhibitors of PDE 2, 3, 4 and 5 indicated that the specificity for PDE3 inhibitors resides in the expressed C-terminal fragment of the enzyme and is not significantly affected by removal of the N-terminus (Table 2).

Immunoprecipitates of cGI-PDEΔ1 and cGI-PDEΔ1Δi containing similar amounts of immunoreactive protein (Figure 4**a**) were compared with respect to their abilities to hydrolyse cAMP and cGMP (Figure 4**b** and 4**c**). Despite its limited capacity for binding [^{32}P]cGMP, no enzyme activity was detected in cGI-PDEΔ1Δi.

Site-directed mutagenesis of the 44 amino acid insert in cGI-PDEΔ1

Alignment of the 44 amino acid inserts of human cardiac PDE3A and rat adipocyte PDE3B showed that fifteen amino acid residues were conserved (Figure 5**a**). Analysis of the sequences of PDE3A and PDE3B according to Garnier *et al.* [24] was used to identify conserved regions with potential structural roles. A probable β-turn was located at the C-terminus of the insert (YGC) and a possible β-turn at the N-terminus (PGL). Two residues in each of these sequences were replaced with alanine residues (Figure 5**a**). A region containing a cluster of negative charges (SDSDS), as well as a conserved downstream glycine residue were also mutated (Figure 5**a**). Photolabelling of these mutants with [^{32}P]cGMP, was unaffected in four of the five examined (Figure 5**b**). In these experiments, western blot analysis of the immunoprecipitates permitted correlation of the extent of photolabelling with the amount of expressed protein used in each sample (Figure 5**c**). Despite the presence of sufficient amounts of expressed protein, the P1A G2A mutant remained very poorly photolabelled relative to the cGI-PDEΔ1 control (Figure 5**b**; compare lanes 2 and 5). Cyclic nucleotide hydrolytic activities of immunoprecipitates of these mutants were compared with those of immunoprecipitates of cGI-PDEΔ1 (Figure 6). The results indicated that both cAMP and cGMP hydrolysis by the P1A G2A and Y42A G43A mutants were markedly

reduced relative to cGI-PDEΔ1 and the other mutants (Figure 6a). The ratio of cAMP to cGMP hydrolysis, at 0.5 μM substrate concentrations, were unchanged by any of these alanine substitutions. Moreover, the inhibitory effect of 0.1 μM lixazinone was very similar for all the mutant enzymes examined and was not diminished in the P1A G2A and Y42A G43A mutants despite their lower enzyme activities (Figure 6b). These results suggest that the 44 amino acid insert may not be critical for inhibition of the enzyme.

DISCUSSION

Purified platelet mRNA and degenerate PCR primers, based on published platelet peptide sequences [16], were used to clone a fragment of platelet PDE3. The nucleotide sequence of this partial clone was identical with a segment of the previously cloned human cardiac enzyme [16], indicating that both platelet and cardiac forms of PDE3 were derived from the same gene (PDE3A). Despite this common origin, fundamental differences exists between the platelet and cardiac enzymes. Platelet PDE3 is cytosolic with a subunit molecular mass of about 110 kDa [7, 21], whereas the cloned cardiac enzyme is larger (126 kDa) and appears to be predominantly membrane-associated [25]. Moreover, the PDE3 inhibitor, vesnarinone, has been reported to distinguish between the PDE3 enzymes from human heart and platelets, preferentially inhibiting the former [26]. To date, two PDE3 genes have been identified [16, 17] and shown to be expressed in a tissue specific manner. As defined in the latest nomenclature [11], the PDE3A gene is expressed in vascular smooth muscle, platelets and cardiac muscle, whereas PDE3B is found predominantly in

adipose and liver cells. Recently, a 74 kDa soluble human placental phosphodiesterase was cloned [27] and found to be identical in sequence to the C-terminus of the human cardiac enzyme. Northern blots and RNAse protection assays identified two transcripts (4.4 kb and 7.6 kb) in human placenta. The smaller transcript was generated from an alternate transcriptional start site and encoded the 74 kDa cytosolic enzyme, whereas the larger 7.6 kb transcript coded for a membrane-associated enzyme. A comparable relationship between the soluble platelet and particulate cardiac enzymes may exist, given that two transcripts have also been detected in human erythroleukemia (HEL) cells, a megakaryocytic human cell line that expresses many platelet proteins [28]. Thus, the presence of an additional transcriptional start site or alternate splicing at the N-terminus of the PDE3A gene could account for the differences observed between the platelet and cardiac isoforms of PDE3. Sequences corresponding to the N-terminus of the cloned membrane-bound cardiac enzyme have very recently been amplified from platelet mRNA [28], but this does not resolve the identity of the N-terminus of the smaller, soluble platelet enzyme.

We have cloned and expressed the C-terminal half of platelet PDE3A (cGI-PDEΔ1) in the *E. coli* strain M15. Since the N-terminus of the platelet cGI-PDE remains to be defined and is unlikely to be identical to that of the cloned cardiac enzyme, N-terminal truncation mutants of platelet cGI-PDE were compared to the full length native platelet enzyme. To facilitate rapid isolation of the undegraded enzymes, immunoprecipitates were isolated and compared with respect to enzyme kinetics and inhibitor sensitivity. Our results demonstrate that K_m values for the hydrolysis of cAMP and cGMP were

significantly lower for the N-terminal deletion mutants than for the intact enzyme. Other groups have also reported on the effects of N-terminal deletions in the PDE3A gene. Pillai *et al.* [29] demonstrated that truncated forms of the cardiac enzyme expressed in yeast hydrolysed cAMP with K_m values *higher* than that of the expressed intact cardiac enzyme. Kasuya *et al.* [27] expressed the 74 kDa placental cGI-PDE in a baculovirus system and reported that, although the K_m value for cAMP was unchanged, that for cGMP was increased to 3.27 ± 1.75 μM. However, in a very recent study of enzymes expressed in Sf9 cells [30], kinetic analysis of two N-terminal deletion mutants of cardiac PDE3 did not demonstrate any significant difference in the K_m for cAMP for these mutants, relative to the intact expressed cardiac enzyme (0.15 - 0.22 μM). There are several possible explanations for the differences between our kinetic results and those reported by others. First, our results were derived from enzymes purified by immunoprecipitation or nickel-resin chromatography and were not assayed in crude homogenates. Thus, it is possible that extracts of yeast or Sf9 cells contain proteins or enzymes capable of modifying the activity of the expressed cGI-PDEs. In addition, the deletion mutants used in our studies are 27 to 77 residues shorter than those discussed above [29,30]. Finally, differences between investigators in terms of the phosphodiesterase assay conditions used (Mg^{2+} vs Mn^{2+}) and the protein expression systems used (yeast vs baculovirus vs *E. coli*) may have contributed to differences in the kinetic results.

Shortly after completion of the present study, Cheung *et al.* [28] reported the isolation of cDNA clones expressing PDE3 sequences from a HEL cell library. The clones

were 100% identical at the nucleotide level to the cardiac enzyme. These investigators contend that N-terminal truncated forms of these clones expressed enyzmes with K_m values that were virtually identical to that of the purified platelet enzyme. Since it is well-established that purification of platelet PDE3 results in the isolation of proteolyzed forms [6,23], it is quite likely that the reported K_m values for 'intact' enzyme are representative of PDE3 fragments. Indeed, the K_m value of 0.2 μM [28] is consistent with our value of 0.18 μM for the truncated enzyme. As previously demonstrated [21], the immunoprecipitation procedure utilized in the present study permits the isolation of non-proteolyzed platelet PDE3 enzyme, which can then be directly compared to various truncation mutants.

The K_m values obtained for hydrolysis of cAMP and cGMP by immunoprecipitates of intact platelet enzyme were 398 ± 50 nM and 252 ± 16 nM, respectively. The respective values given in the literature for these two parameters using the platelet enzyme are quite variable, for example: 0.18 μM and 0.02 μM [6], 0.20 μM and 0.30 μM [23] and 1.0 μM for cAMP [31]. As discussed above, it is likely that some of these published values represent the enzyme activities of fragments rather than intact cGI-PDE. PDE3 is not only known to have a high affinity for cGMP, but also a low ratio of the V_{max} for cGMP to that for cAMP [11]. We therefore compared the cGMP hydrolytic abilities of our N-terminal deletion mutants with those of the intact platelet enzyme. Our results indicated that as N-terminal sequences were deleted, a progressively lower K_m for cGMP was generated and a concomitant three-fold increase in the ratio of V_{max} for cGMP/V_{max} for cAMP was detected. Thus, deletion of

sequences from the N-terminus of platelet PDE3 increased the activity of the enzyme towards cGMP relative to cAMP. This implies that directly or indirectly, the N-terminal sequences in part determine the substrate specificity of the enzyme.

A distinguishing feature of the PDE3 gene family is the presence of a 44 amino acid insert located within the catalytic domain. The function of this insert, which bears no relationship to other sequences currently in the protein databases is unknown, though it initially seemed reasonable to speculate that it had a role in the unique properties of the PDE3 family. Although removal of this insert destroyed enzyme activity, a limited ability to bind cGMP, as detected by photolabelling, was retained. Structural analysis of the 44 amino acid inserts of human cardiac and rat adipocyte enzymes [24] identified a high potential for a β-turn at the C-terminus of the inserts and a possible β-turn at the N-terminus. Site-directed mutagenesis of pairs of amino acids in either of these putative β-turns markedly decreased enzyme activity, though cGMP binding, as determined by photoaffinity labelling, was only inhibited by mutagenesis of the N-terminal insert residues. Mutagenesis of a conserved cluster of negatively charged residues in the centre of the insert was without effect. Although our results did not identify any specific functional role for amino acid residues in the insert, it seems clear that the insert is required to preserve an effective catalytic domain structure.

The effects of the PDE3 inhibitor lixazinone, on the cAMP hydrolytic activities of intact platelet cGI-PDE and the N-terminal truncation mutants, were studied. Our results demonstrated that lixazinone increased the K_m of the intact platelet enzyme, but

left the V_{max} largely unaffected, indicating that it is a competitive inhibitor. In contrast, mixed inhibition with roughly equal competitive and uncompetitive components was seen with the cGI-PDEΔ1 deletion mutant. The previously reported inhibitory effect of lixazinone on platelet cGI-PDE purified by DEAE-cellulose chromatography [32] also indicated a mixed pattern of inhibition, similar to that obtained for the N-terminal deletion mutant, cGI-PDEΔ1. Since purification of PDE3 by this method is known to generate proteolytic fragments, it is possible that the inhibitory profile reported in [32] is representative of a PDE3 fragment rather than the intact platelet enzyme. The high inhibitory potency of lixazinone is due to the molecular interaction between the compound and a bulk-tolerant, lipophilic region of the enzyme [33]. Since inhibition by this compound changes from predominantly competitive, for the intact enzyme, to mixed in the deletion mutant, it is possible that removal of the N-terminus provides the inhibitor with access to an alternate binding site.

Regardless of the mechanism, it is clear that the N-terminal deletion mutants retain the capacity of the intact enzyme to respond selectively to PDE3 inhibitors. Moreover, no changes in inhibition of the enzyme by lixazinone were seen in site-directed mutants of the 44-amino acid insert. Thus, the characteristic inhibitor profile of PDE3 likely reflects the structure of the catalytic domain, though C-terminal sequences which were not deleted in this study, could also be involved.

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REFERENCES

1 Haslam, R.J., Davidson, M.M.L., Davies, T., Lynham, J.A. and McClenaghan, M.D. (1978) in Adv. Cycl. Nucleotide Res. **9**, p. 533-552, Raven Press, New York

2 Zucker, M.B. and Nachmias, V.T. (1985) Arteriosclerosis **5**, 2-18

3 Kroll, M.H. and Schafer, A.I. (1989) Blood **74**, 1181-1195

4 Siess, W. (1989) Pharmacol. Rev. **69**, 58-178

5 Hidaka, H. and Asano, T. (1976) Biochim. Biophys. Acta **429**, 485-497

6 Grant, P.G. and Colman, R.W. (1984) Biochemistry **23**, 1801-1807

7 Macphee, C.H., Harrison, S.A. and Beavo, J.A. (1986) Proc. Natl. Acad. Sci. U.S.A. **83**, 6660-6663

8 Hamet, P. and Tremblay, J. (1988) Methods Enzymol. **159**, 710-722

9 Robichon, A. (1991) J. Cell. Biochem. **47**, 147-157

10 Grant, P.G., Mannarino, A.F. and Colman, R.W. (1990) Thromb. Res. **59**, 105-119

11 Beavo, J.A. (1995) Physiol. Rev. **75**, 725-748

12 Sheth, S.B. and Colman, R.W. (1995) Platelets **6**, 61-70

13 Maurice, D.H. and Haslam, R.J. (1990) Mol. Pharmacol. **37**, 671-681

14 Charbonneau, H., Beier, N., Walsh, K.A. and Beavo, J.A. (1986) Proc. Natl. Acad. Sci. U.S.A. **83**, 9308-9312

15 Manganiello, V.C., Murata, T., Taira, M., Belfrage, P. and Degerman, E. (1995) Arch. Biochem. Biophys. **322**, 1-13

16 Meacci, E., Taira, M., Moos, M., Smith, C.J., Movsesian, M.A., Degerman, E., Belfrage, P. and Manganiello, V. (1992) Proc. Natl. Acad. Sci. U.S.A. **89**, 3721-3725

17 Taira, M., Hockman, S.C., Calvo, J.C., Taira, M., Belfrage, P. and Manganiello, V.C. (1993) J. Biol. Chem. **268**, 18573-18579

18 Manganiello, V.C., Taira, M., Degerman, E. and Belfrage, P. (1995) Cell. Signalling **7**, 445-455

19 Mustard, J.F., Perry, D.W., Ardlie, N.G. and Packham, M.A. (1972) Br. J. Haematol. **22**, 193-204

20 Landt, O., Grunert, H-P. and Hahn, U. (1990) Gene **96**, 125-128

21 Tang, K.M., Sherwood, J.L. and Haslam, R.J. (1993) Biochem. J. **294**, 329-333

22 Davis, C.W. and Daly, J.W. (1979) J. Cycl. Nucleotide Res. **5**, 65-74

23 Degerman, E., Moos, M., Rascon, A., Vasta, V., Meacci, E., Smith, C.J., Lindgren, S., Andersson, K-E., Belfrage, P. and Manganiello, V.C. (1994) Biochim. Biophys. Acta **1205**, 189-198

24 Garnier, J., Osguthorpe, D.J. and Robson, B. (1978) J. Mol. Biol. **120**, 97-120

25. Smith, C.J., Krall, J., Manganiello, V.C. and Movsesian, M.A. (1993) Biochem. Biophys. Res. Commun. **190**, 516-521

26 Masuoka, H., Ito, M., Sugioka, M., Kozeki, H., Konishi, T., Tanaka, T. and Nakano, T. (1993) Biochem. Biophys. Res. Commun. **190**, 412-417

27. Kasuya, J., Goko, H. and Fujita-Yamaguchi, Y. (1995) J. Biol. Chem. **270**, 14305-14312

28 Cheung, P.P., Xu, H.,McLaughlin, M.M., Ghazaleh, F.A., Livi, G.P., and Colman R.W. (1996) Blood **88**, 1321-1329

29 Pillai, R., Staub, S.F. and Colicelli, J. (1994) J. Biol. Chem. **269**, 30676-30681

30 Leroy, M.-J., Degerman, E., Taira, M., Murata, T., Wang, L.H., Movsesian, M.A., Meacci, E., Manganiello, V.C. (1996) Biochemistry **35**, 10194-10202

31 Sheth, S. and Colman, R.W. (1994) FASEB J. **8**, A83

32 Alvarez, R., Banerjee, G.L., Bruno, J.J., Jones, G.L., Liittschwager, K., Strosberg, A.M. and Venuti, M.C. (1986) Mol. Pharmacol. **29**, 554-560

33 Venuti, M.C., Stephenson, R.A., Alvarez, R., Bruno, J.J. and Strosberg, A.M. (1988) J. Med. Chem. **31**, 2136-2145

Table 1 **Comparison of the kinetic properties of expressed cGI-PDE deletion mutants with those of the intact enzyme**

Intact platelet PDE3 was immunoprecipitated from platelet supernatant and mutant PDEs from bacterial supernatants, as described in the Experimental section. K_m and V_{max} values were calculated by non-linear regression analysis using explict weighting based on results obtained in triplicate assays. IC_{50} values were determined using 0.5 μM cAMP as substrate. To eliminate small amounts of PDE5 contaminating platelet cGI-PDE immunoprecipitates, 10 μM zaprinast was added to assays of this enzyme. Results are means ± S.E. from the numbers of separate enzyme preparations shown in parentheses.

Enzyme	K_m (cAMP) (nM)	K_m (cGMP) (nM)	$\frac{V_{max}\text{ (cGMP)}}{V_{max}\text{ (cAMP)}}$	IC_{50} for lixazinone (nM)	IC_{50} for milrinone (nM)
cGI-PDEΔ1	182 ± 12(3)	153 ± 12(3)	0.37 ± 0.05(3)	186 ± 26(4)	1148 ± 73(3)
cGI-PDEΔ2	131 ± 17(3)	99 ± 1(3)	0.49 ± 0.04(3)	165 ± 41(1)	1207 ± 151(1)
Platelet cGI-PDE	398 ± 50(3)	252 ± 16(3)	0.16 ± 0.01(3)	53 ± 10(4)	474 ± 92(3)

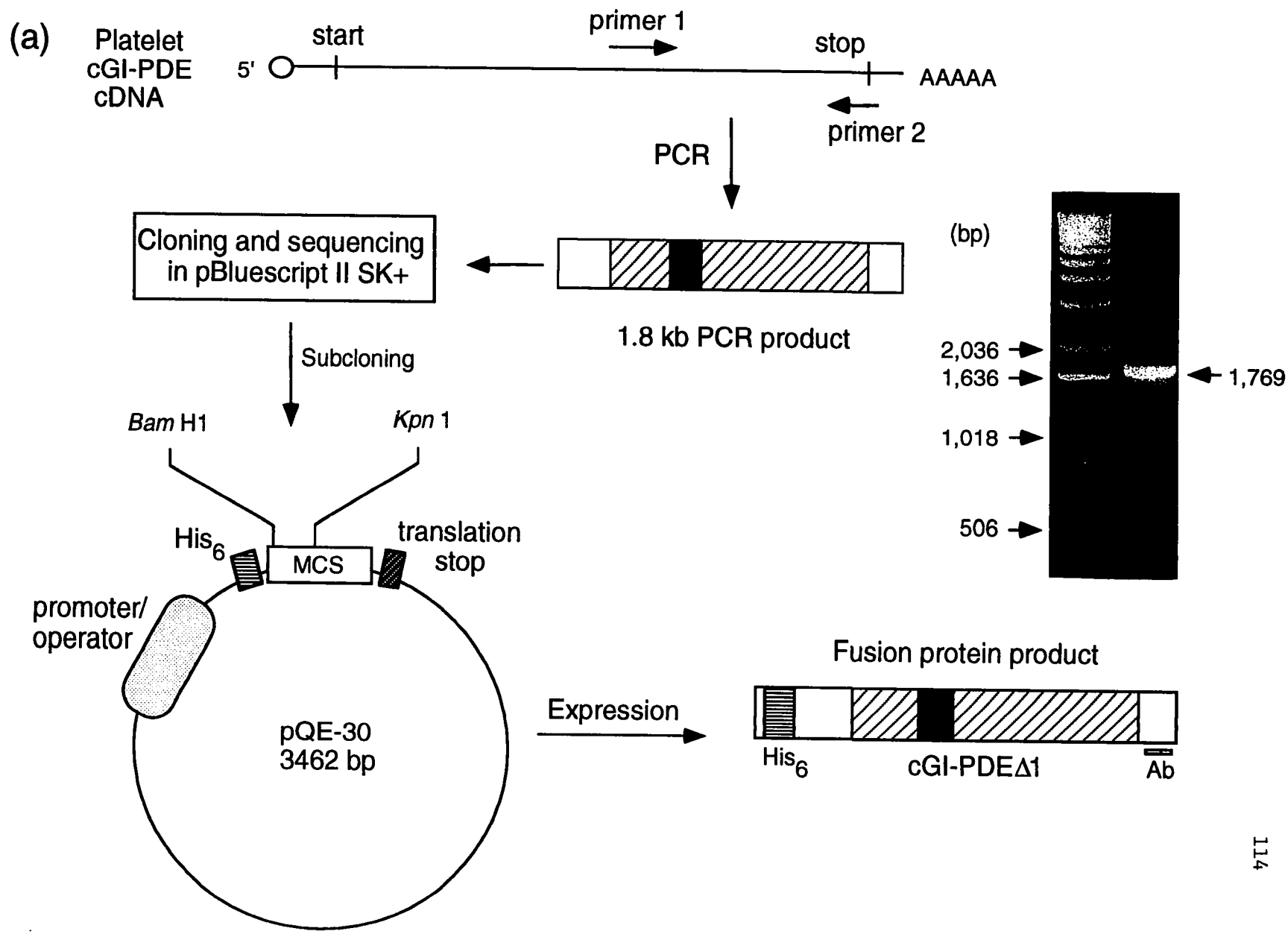
Table 2 **Specificity of the effects of PDE inhibitors on cGI-PDEΔ1, cGI-PDEΔ2 and platelet cGI-PDE**

Immunoprecipitates of cGI-PDE from platelets and *E. coli* expressing cGI-PDE deletion mutants were incubated with 0.5 μM cAMP and the indicated PDE inhibitors for 5 min at 30 °C. Results are means ± S.E. from assays carried out in triplicate.

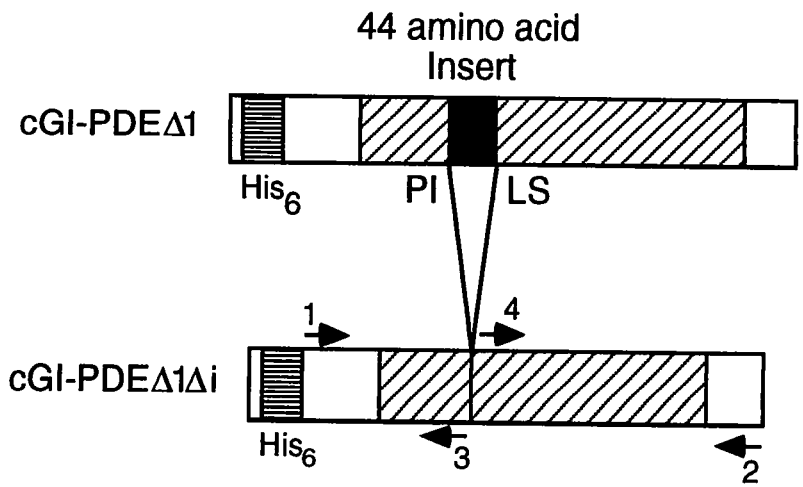
	Inhibition of cAMP hydrolysis (%)		
Inhibitor	cGI-PDEΔ1	cGI-PDEΔ2	Platelet cGI-PDE
Milrinone (10 μM)	84 ± 1	82 ± 1	86 ± 1
Zaprinast (10 μM)	1 ± 6	6 ± 5	0 ± 3
EHNA (20 μM)	-4 ± 6	-2 ± 5	6 ± 3
Rolipram (10 μM)	-5 ± 5	-1 ± 6	5 ± 6

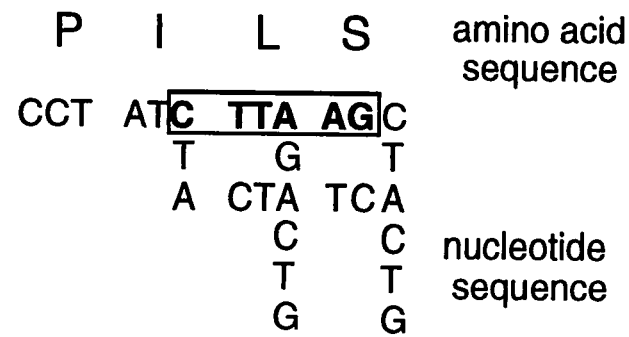
Figure 1 Cloning strategy for the generation of platelet cGI-PDE deletion mutants and the specific removal of the 44 amino acid insert from the catalytic domain

(**a**) A 1.8 kb region of platelet cGI-PDE was amplified using RT-PCR and platelet mRNA. The PCR product (see photograph of agarose gel), which coded for the complete catalytic domain of cGI-PDE, was cloned into pBluescript II SK^+ for sequencing and then subcloned into the bacterial expression vector pQE-30. The His_6-tagged protein, representing a truncated form of the human platelet cGI-PDE (cGI-PDEΔ1), was expressed to high levels in *E.coli.* (**b**) The 44 amino acid insert characteristic to the PDE3 family was specifically removed from cGI-PDEΔ1 using a PCR approach, to give cGI-PDEΔ1Δi. Primers 1 and 3 were used to amplify the region upstream of the insert and primers 2 and 4 to amplify the downstream region. By incorporating an *Afl*II restriction site into primers 3 and 4, the boundary formed by the ligation of these two regions produced the required PILS amino acid sequence. Diagonal shading, catalytic domain; solid area, 44 amino acid insert; horizontal shading, His_6 sequence from vector; Ab, antibody binding site.


(a)
Platelet cGI-PDE cDNA
5'
start
primer 1
stop
AAAAA
primer 2
PCR
1.8 kb PCR product
Cloning and sequencing in pBluescript II SK+
Subcloning
(bp)
2,036
1,636
1,018
506
1,769
Bam H1
Kpn 1
His6
MCS
translation stop
promoter/ operator
pQE-30
3462 bp
Expression
Fusion protein product
His6
cGI-PDEΔ1
Ab

(b)





primer 4

5 '- ATC TTA AGC GGG AAT ATC CCT GCC TTG GAG - 3 '

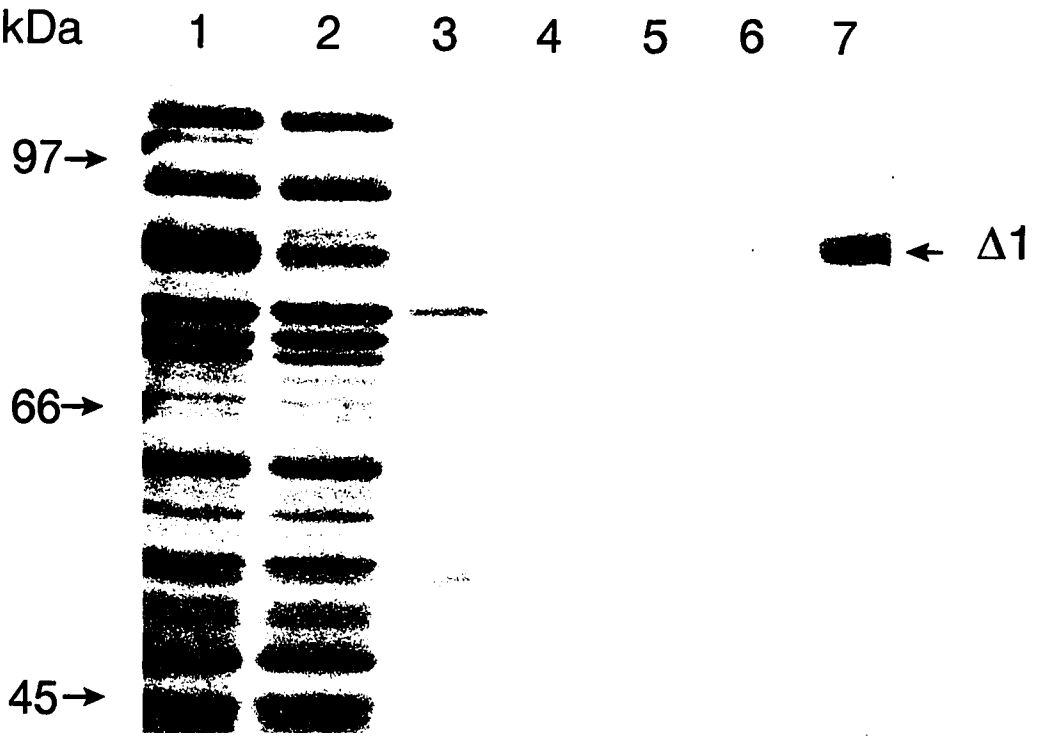
3' - ACC ATA GAA TGA TGT GTC GGA TAG AAT TCG CC - 5 '

primer 3 *Afl* II

Figure 2 Purification of cGI-PDE deletion mutants by nickel-resin column chromatography or immunoprecipitation

E.coli expressing cGI-PDE deletion mutants were harvested and lysed by sonication. After centrifugation at 100,000 x *g*, the His_6-tagged proteins were purified either by nickel-resin column chromatography or immunoprecipitation. (**a**) Soluble protein from *E.coli* expressing cGI-PDEΔ1 (lane 1) was loaded onto a nickel-resin column to bind histidine-containing proteins. After removal of unbound protein (lane 2) and a series of increasingly stringent washes (lanes 3-5), purified protein was eluted from the column (lanes 6 and 7) (see Experimental section). Protein was analyzed by SDS/PAGE; a Coomassie Blue-stained gel is shown. (**b**) Immunoblots of supernatants from bacteria expressing cGI-PDE deletion mutants are shown (lanes 1-3). Purification of the expressed proteins from the supernatants was achieved by immunoprecipitation and the immunoprecipitates were then analyzed by immunoblotting (lanes 4-6). Lanes 1 and 4, cGI-PDEΔ1 (Δ1); lanes 2 and 5, cGI-PDEΔ1Δi (Δ1Δi); lanes 3 and 6, cGI-PDEΔ2 (Δ2). The positions of protein standards are shown on the left (see Experimental section).

(a)



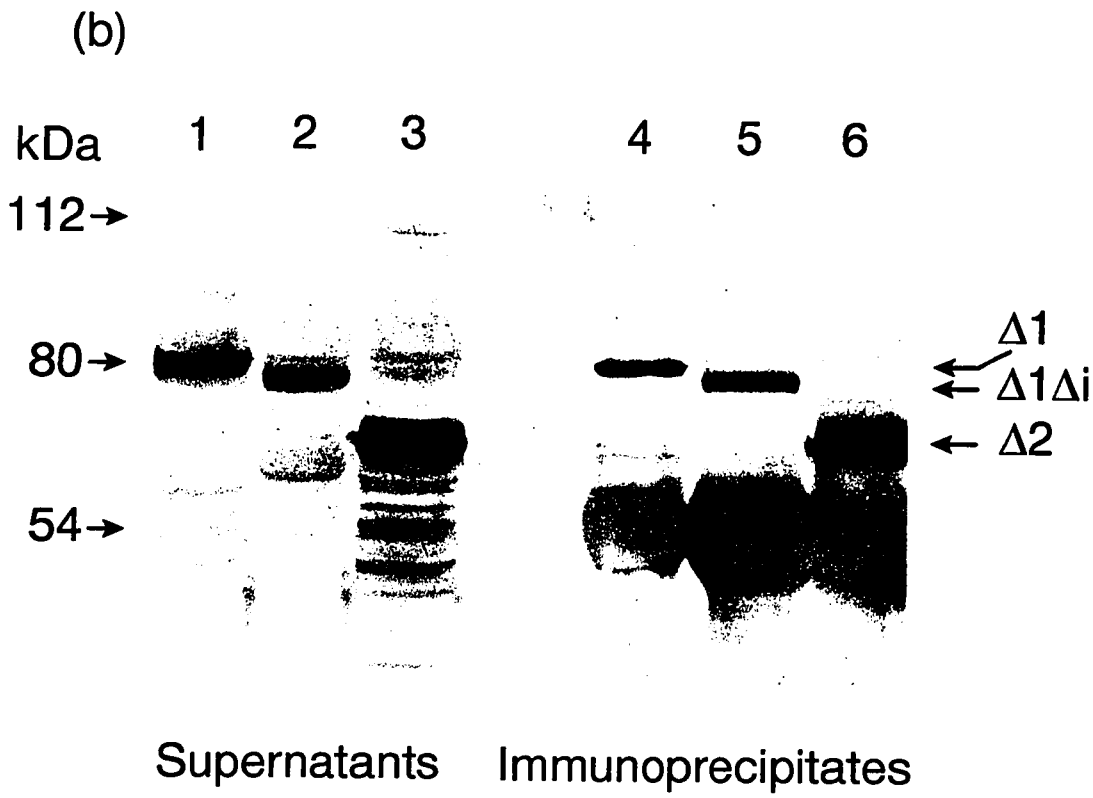
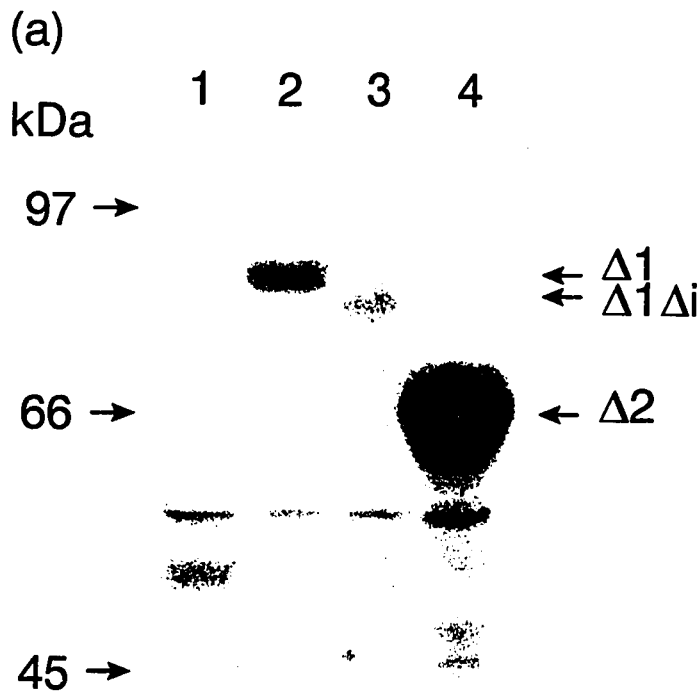

(b)
kDa
1
2
3
4
5
6
112→
80→
54→
Δ1
Δ1Δi
Δ2
Supernatants
Immunoprecipitates

Figure 3 Photoaffinity labelling of extracts of *E. coli* expressing cGI-PDE deletion mutants

(a) Bacterial supernatants were photolabelled with [^{32}P]cGMP as described under Experimental. Lane 1, Control (*E. coli* transformed with vector alone); lane 2, cGI-PDEΔ1; lane 3, cGI-PDEΔ1Δi; lane 4, cGI-PDEΔ2. The positions of protein standards are shown on the left (see Experimental section). **(b)** Effects of PDE3 inhibitors on the photoaffinity labelling of bacterial supernatants containing cGI-PDEΔ1 (Δ1) and cGI-PDEΔ2 (Δ2). Lane 1, no inhibitor; lane 2, 10 μM lixazinone; lane 3, 40 μM milrinone; lane 4, 40 μM cGMP.


(a)
1
2
3
4
kDa
97
66
45
Δ1
Δ1Δi
Δ2

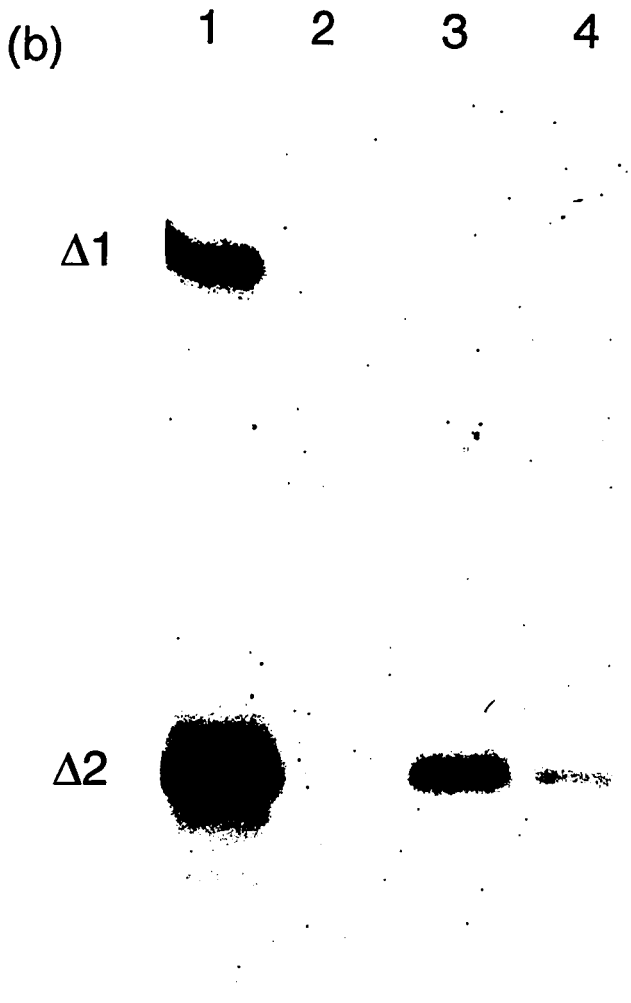

(b)
1
2
3
4
Δ1
Δ2

Figure 4 Phosphodiesterase activities of expressed cGI-PDEΔ1 and cGI-PDEΔ1Δi

The mutant enzymes were immunoprecipitated from bacterial extracts, as described under Experimental and assayed for phosphodiesterase activities. (**a**) Quantitation of immunoprecipitated enzymes by immunoblotting. Lanes 1-3, 25, 50 and 100 μl of cGI-PDEΔ1; lane 4, prestained 80 kDa marker; lanes 5-7, 25, 50 and 100 μl of cGI-PDEΔ1Δi. (**b**) cAMP phosphodiesterase activities. Immunoprecipitated enzymes (50 μl aliquots) were incubated with 0.5 μM cAMP for 5 min at 30 °C. (**c**) cGMP phosphodiesterase activities. Immunoprecipitated enzymes (50 μl aliquots) were incubated with 0.5 μM cGMP for 10 min at 30 °C. Results are means ± S.E. from triplicate assays.

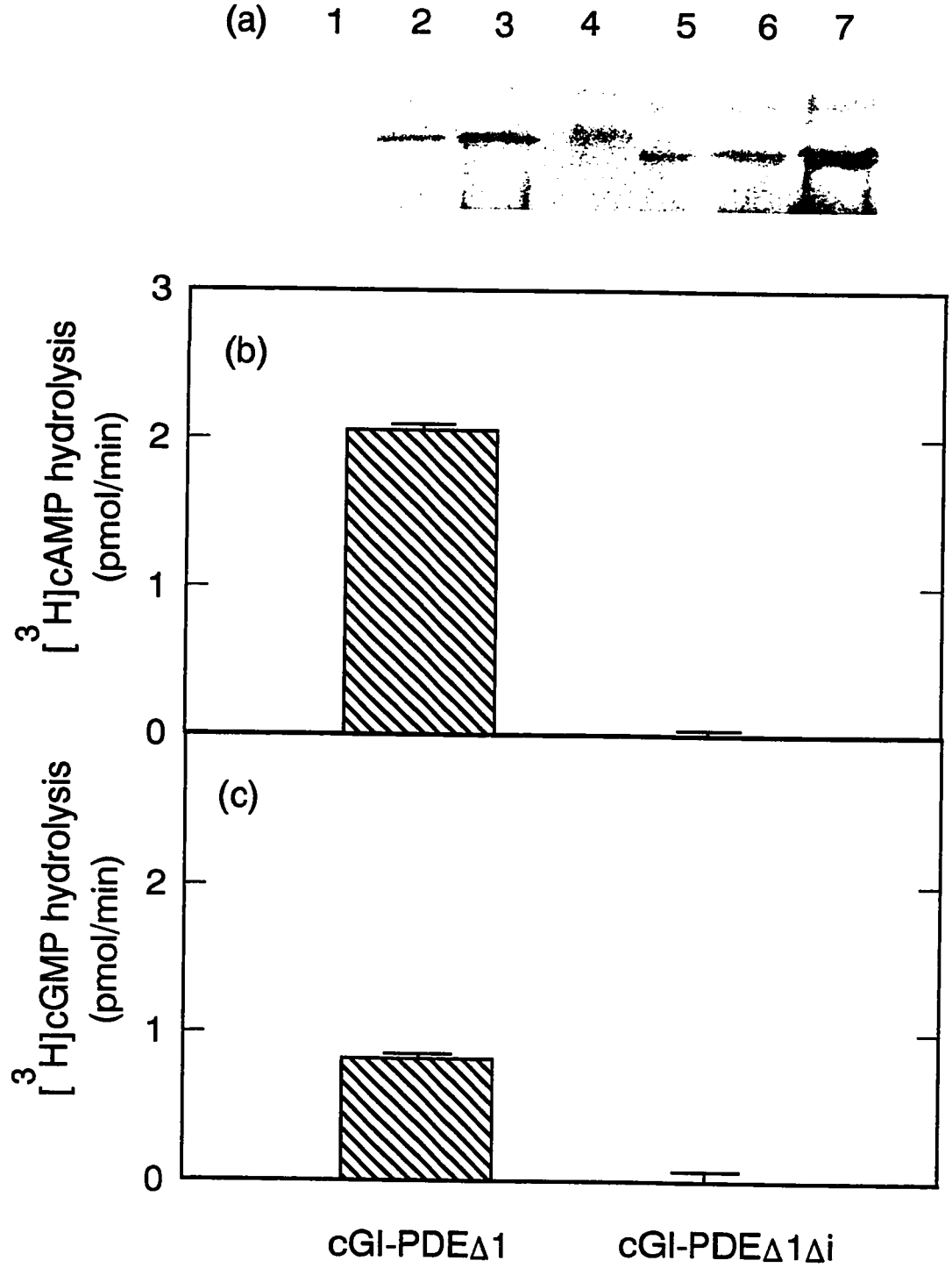
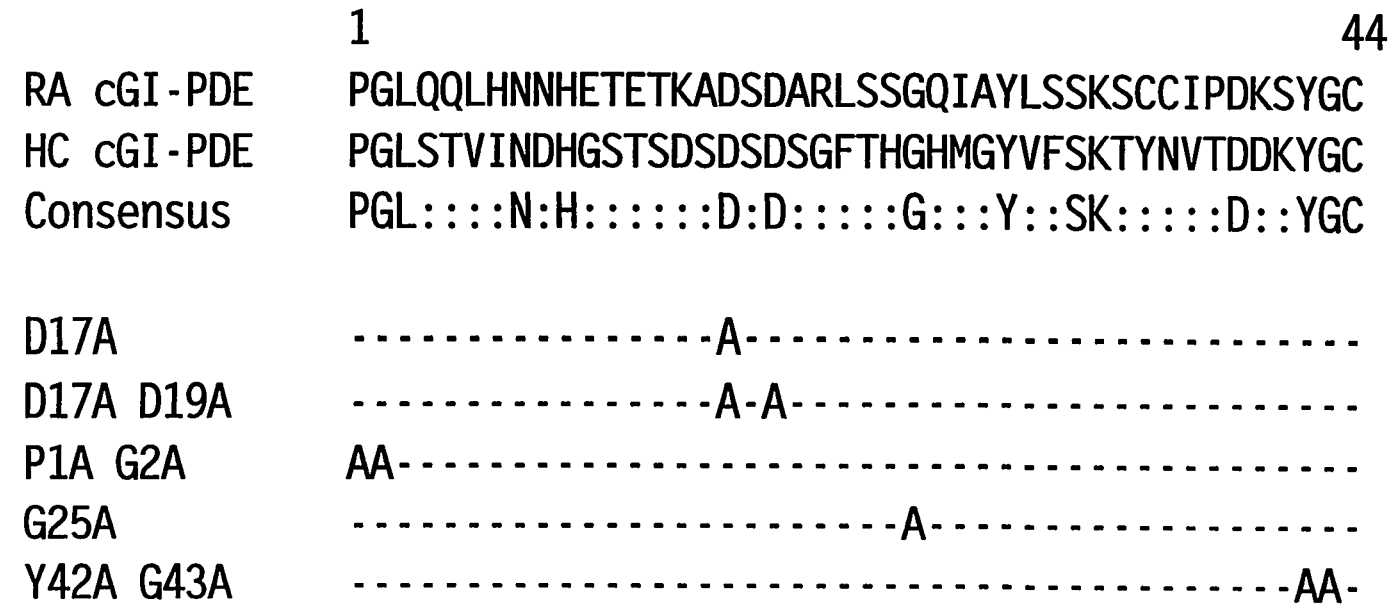

(a)
1
2
3
4
5
6
7
(b)
[3H]cAMP hydrolysis (pmol/min)
3
2
1
0
(c)
[3H]cGMP hydrolysis (pmol/min)
2
1
0
cGI-PDEΔ1
cGI-PDEΔ1Δi

Figure 5 Site-directed mutagenesis of the 44 amino acid insert within the catalytic domain of cGI-PDEΔ1

(**a**) The 44 amino acid inserts from rat adipocyte PDE3B (RA cGI-PDE) and human cardiac PDE3A (HC cGI-PDE) were aligned to identify conserved amino acid residues. Selected residues were replaced with alanine by site-directed mutagenesis of cGI-PDEΔ1. The mutants shown were cloned and expressed in *E.coli*, (see Experimental section). (**b**) Bacterial supernatants containing expressed mutants of cGI-PDEΔ1 were photolabelled with [^{32}P]cGMP and the protein was analyzed by SDS/PAGE. An autoradiograph is shown. (**c**) Immunoprecipitates from the same supernatants were analyzed by immunoblotting. The supernatants used in (**b**) and (**c**) were from the following sources: lane 1, *E.coli* transformed with vector alone; lane 2, *E.coli* expressing cGI-PDEΔ1; lanes 3-7, *E.coli* expressing the following cGI-PDEΔ1 insert mutants: 3, D17A; 4, D17A D19A; 5, P1A G2A; 6, G25A; 7, Y42A G43A. Mutated residues are numbered according to their positions in the 44 amino acid insert (see **a**).

(a)


1
44
RA cGI-PDE PGLQQLHNNHETETKADSDARLSSGQIAYLSSKSCCIPDKSYGC
HC cGI-PDE PGLSTVINDHGSTSDSDSDSGFTHGHMGYVFSKTYNVTDDKYGC
Consensus PGL::::N:H::::::D:D:::::G:::Y::SK:::::D::YGC
D17A ----------A----------
D17A D19A ----------A-A----------
P1A G2A AA----------
G25A ----------A----------
Y42A G43A ----------AA-

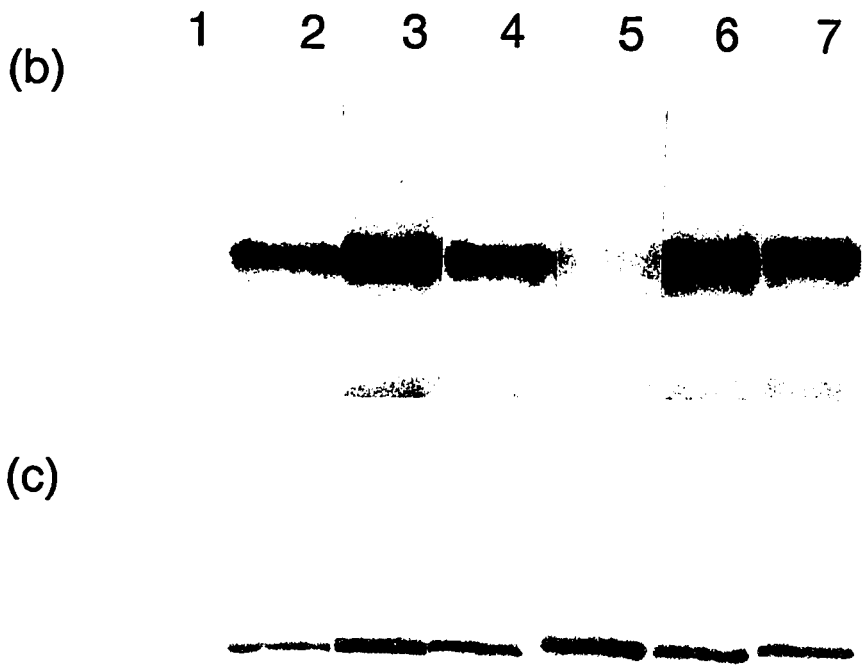
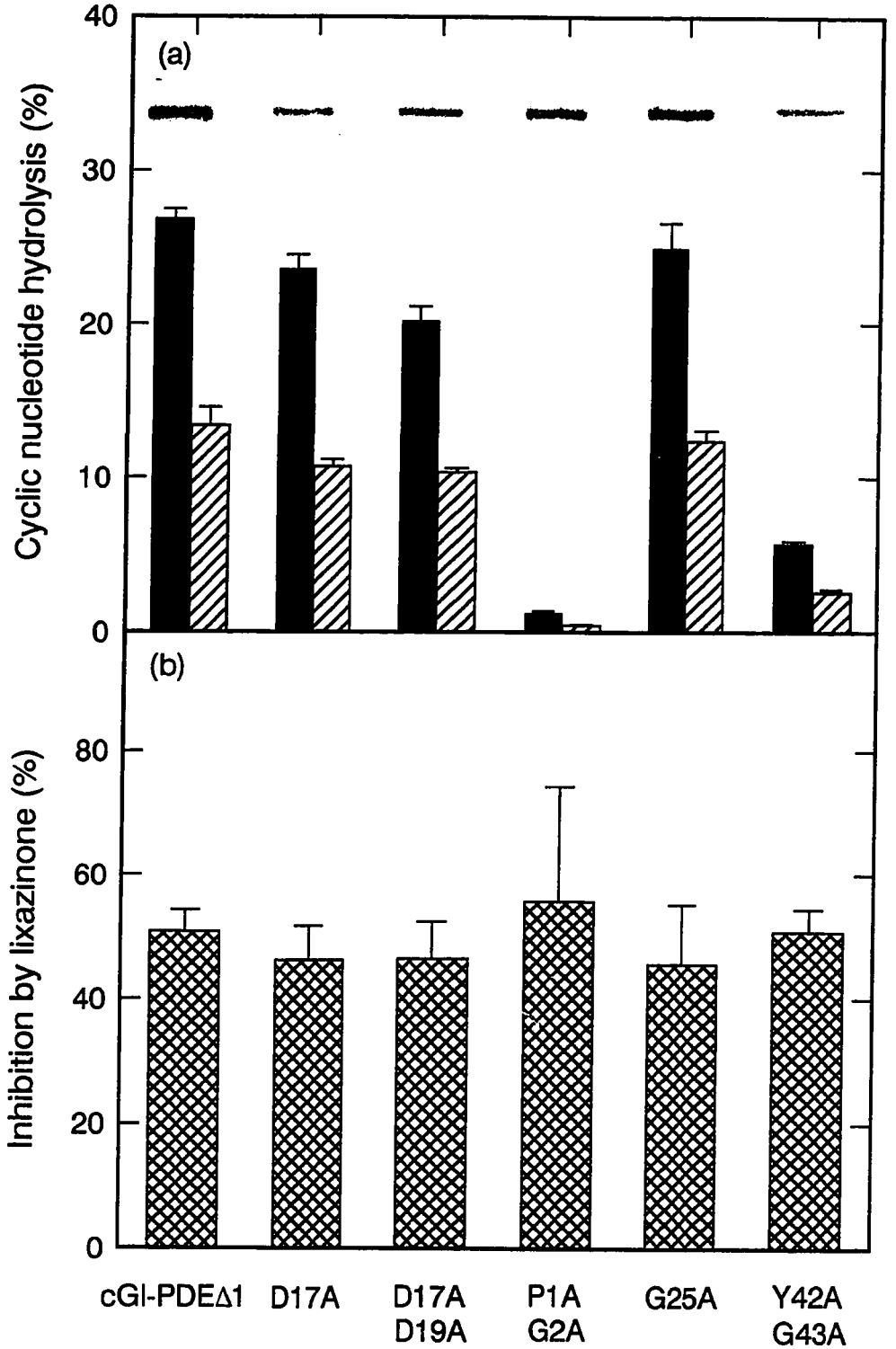

1 2 3 4 5 6 7
(b)
(c)

Figure 6 Enzymatic properties of site-directed mutants of cGI-PDEΔ1

The expressed mutant enzymes were immunoprecipitated from bacterial extracts. (**a**) The enzyme activities of the immunoprecipitates were determined in 5 min incubations at 30 °C with 0.5 μM cAMP (solid bars) and 0.5 μM cGMP (striped bars). The amounts of the different immunoprecipitated enzymes assayed were compared by immunoblotting (see insert). (**b**) Inhibition of cAMP hydrolysis by 0.1 μM lixazinone was determined in 5 min incubations at 30 °C with 0.5 μM cAMP. All results are means ± S.E. from three identical assays.


(a)
Cyclic nucleotide hydrolysis (%)
40
30
20
10
0
(b)
Inhibition by lixazinone (%)
80
60
40
20
0
cGl-PDEΔ1
D17A
D17A
D19A
P1A
G2A
G25A
Y42A
G43A

CHAPTER 5

GENERAL DISCUSSION

The underlying theme of my studies has been to investigate the molecular mechanisms of action of cGMP in platelets. Although it is well-established that PKG is an important site of action of cGMP (Walter, 1989), other targets of cGMP, such as PDE3, have also been shown to make significant contributions to the regulation of platelet function (Maurice and Haslam, 1990a). Thus, I have developed a method to identify cGMP-binding proteins in platelets and other cells as an initial first step toward developing a better understanding of the multiple sites of action of cGMP.

5.1 Photoaffinity Labelling Technique

The technique of photoaffinity labelling itself is relatively straight-forward and involves the covalent attachment of a radiolabelled ligand to a specific binding site during ultraviolet irradiation (Kiefer et al., 1970). In general, the molecule to be bound contains a functional group which can be activated by irradiation to form a covalent bond with adjacent substituents. Although many natural ligands may require chemical modification (e.g. as aryl azides) to be responsive to irradiation, the photolabelling studies undertaken in this thesis utilized unmodified [^{32}P]cGMP to label cellular proteins directly. This process is thought to involve photoactivation of aromatic amino acids (Trp or Tyr) within the cyclic nucleotide binding site rather than modification of cGMP (Antonoff and Ferguson, 1978).

Direct photoaffinity labelling of K30a lymphoma cell extracts by [^{32}P]cGMP has been previously reported by Groppi et al. (1983). Thus, the R_I subunit of PKA and an approximately 106 kDa subunit of PDE3 were labelled in the extracts of K30a mutant cells, which overexpress the latter enzyme. All other reported studies involving photolabelling by [^{32}P]cGMP have been conducted with purified proteins. For example, Stroop et al. (1989) photolabelled purified bovine cardiac PDE2 with [^{32}P]cGMP and demonstrated the presence of two cGMP-binding sites by cyanogen bromide cleavage of the photolabelled protein. Characterization of the purified bovine lung PDE5 enzyme was also accomplished by labelling with [^{32}P]cGMP (Thomas et al., 1990). Despite a preliminary report that PDE5 could be photolabelled by [^{32}P]cGMP in platelets (Walseth et al., 1985), the 93 kDa protein reported by these workers was not detected in our experiments. Neither addition of a compound (IBMX) that enhances cGMP binding by PDE5, nor omission of the PDE5 inhibitor, zaprinast, was sufficient to permit detection of PDE5. The reason for this discrepancy is not clear. However, since the efficiency with which ^{32}P is incorporated into different cGMP-binding proteins may vary and could be very low, it is possible that the conditions utilized in this study did not permit detectable labelling of PDE5.

5.1.1 cGMP-binding proteins in platelet extracts

A general method of labelling cGMP-binding proteins in crude cell extracts was developed and is described in Chapter 2. [^{32}P]cGMP, rather than a labelled analog, was used to photolabel platelet proteins to help to identify all those that bind cGMP.

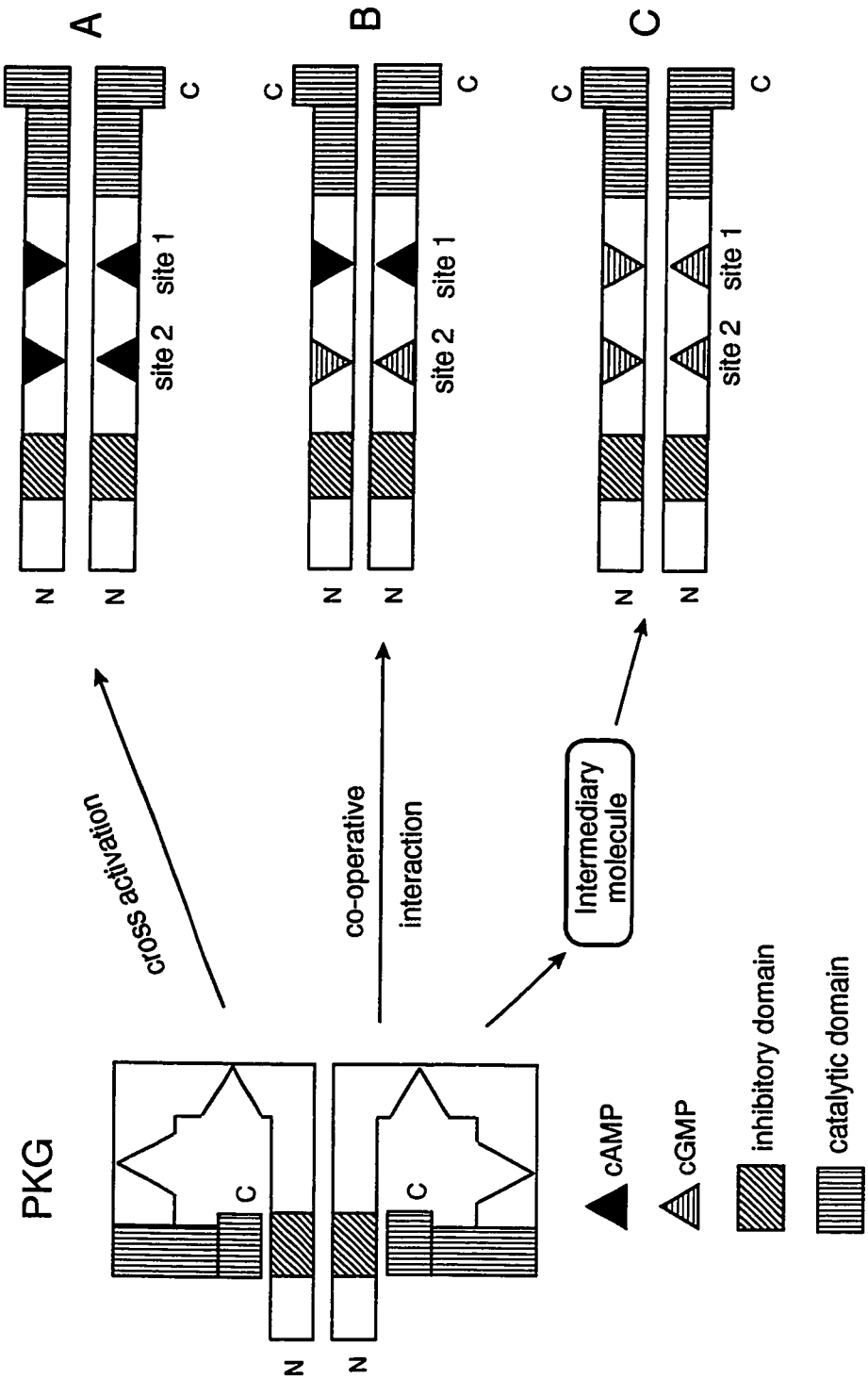
At least five proteins (110, 80, 55, 49 and 38 kDa) were labelled in the platelet soluble fraction and four (80, 65, 49 and 38 kDa) in the membrane fraction. Optimal labelling of the 110 kDa species was achieved in the presence of Mg^{2+} and zaprinast. Omission of these components could explain why Walseth et al. (1985) did not report the presence of a 110 kDa photolabelled species in platelets. Since labelling of the 110 kDa protein was selectively inhibited by PDE3 inhibitors (cilostamide and milrinone) and the labelled protein was specifically immunoprecipitated by an anti-PDE3 antibody, it clearly represents the human platelet PDE3 enzyme. Although [^{32}P]cGMP has been previously shown to photolabel a PDE3 variant in a mutant S49 lymphoma cell line selected for resistance to high cAMP concentrations (Groppi et al., 1983), this is the first demonstration of PDE3 photolabelling in any other cell.

An 80 kDa photolabelled species was detectable in platelet cytosol but was more prominently labelled in the membrane fraction. This protein was identified as Type Iα PKG by immunoprecipitation with bovine anti-PKG antibody and by inhibition of photolabelling with cGMP analogues. In addition, the 80 kDa protein was demonstrated to co-migrate on SDS-PAGE with the purified photolabelled bovine lung enzyme. Although cGMP, 8-bromo-cGMP and Rp-8-bromo-cGMPS inhibited photolabelling, cAMP and Sp-cAMPS greatly stimulated labelling of the 80 kDa protein. Walseth et al. (1985) briefly mentioned that cAMP enhanced the labelling of a 78 kDa species, presumably the protein that we have identified as PKG.

This ability of cAMP to potentiate greatly the labelling of PKG by [^{32}P]cGMP suggests the possibility of a novel co-operative interaction between cAMP and cGMP (see figure 1). Thus, it is possible that the direct association of cAMP with PKG could not only cause simple cross-activation (Francis and Corbin, 1994)(see figure 1A), but could also enhance cGMP binding and thus act synergistically with cGMP to enhance PKG activity (see figure 1B). However, synergistic effects of cAMP and cGMP on purified PKG have not been detected (Corbin et al., 1986) and were not observed in the present experiments, in which cAMP *decreased* photolabelling of purified PKG by [^{32}P]cGMP (see Chapter 2). Autophosphorylation is a common mechanism of regulation of many protein kinases and has been shown to initiate PKG activation and increase its affinity for cAMP and cGMP (Landgraf et al., 1986; Smith et al., 1996). To test whether the action of cAMP depends on autophosphorylation of the enzyme, purified PKG could be treated with cAMP in the presence of ATP and monitored for enhancement of photolabelling and of cGMP-stimulated PKG activity. More plausibly, in view of the evidence (see Chapter 2) that the action of cAMP requires platelet membranes, cAMP could activate an intermediary factor (e.g. PKA) which might then stimulate cGMP binding and activation of the enzyme (see figure 1C). To identify a putative intermediary molecule, purified PKG could be incubated with cAMP and candidate membrane proteins in an attempt to regenerate the effect of cAMP on photoaffinity labelling. The functional significance of the stimulation of cGMP-binding to PKG by cAMP is of considerable interest and could be investigated by monitoring the effect of cAMP added to platelet lysate or membranes on the

Figure 1 Putative models of cAMP action on PKG

Three possible mechanism by which cAMP could influence PKG activity are illustrated. In model A, cAMP directly associates with the cyclic nucleotide binding sites to cross-activate PKG. A co-operative interaction mechanism shown in model B, suggests that binding of cAMP to site 1 (high affinity site) enhances binding of cGMP to site 2 (low affinity site) to increase PKG activity synergistically. Finally, model C postulates that cAMP activates an intermediary molecule which subsequently stimulates cGMP binding and PKG activation. The parallel model for the topological structure of PKG is based on figure by Butt et al. (1993).


PKG
C
N
N
cross activation
co-operative
interaction
Intermediary
molecule
A
B
C
C
N
site 2
site 1
cAMP
cGMP
inhibitory domain
catalytic domain

enzyme activity of immunoprecipitated PKG. Thus, the effect of different concentrations of cAMP and cGMP on the phosphorylation of a synthetic substrate *in vitro* (e.g. Colbran et al., 1992), could provide valuable insights into the possible interactions between cAMP and cGMP *in vivo*.

The 49 and 55 kDa photolabelled species correspond in their molecular masses to the R_I and R_{II} regulatory subunits of PKA; the latter was found exclusively in the cytosol, whereas large amounts of the former were membrane-bound. The molecular masses and subcellular distributions of these proteins were identical to those proteins previously labelled in platelet fractions by 8-azido-[^{32}P]cAMP (Salama and Haslam, 1981). In many tissues, a large proportion of the Type II PKAs are associated with the membrane through interactions with anchoring proteins known as AKAPs (reviewed by Francis and Corbin, 1994). These anchoring proteins contain acidic motifs, which bind to the basic residues present in the regulatory subunit of the Type II enzyme. Thus, AKAPs function as intracellular anchors that tether the holoenzyme to specific subcellular sites. This localization of PKA in close proximity to certain substrates would facilitate a rapid response to any incoming cAMP signal. However, in platelets, the Type II isoform of PKA is cytosolic, whereas much of the Type I isoform is membrane-bound (Salama and Haslam, 1984). A similar distribution of the Type I enzyme has also been reported in human erythrocyte plasma membranes (Rubin, 1979) and in synaptic membranes (Walter, 1978). Although it is clear that AKAPs mediate the distribution of Type II enzyme in most cells, little is known about

the mechanisms by which Type I PKA can be directed to the membrane. It is possible that Type II AKAPs are absent in certain cell types, such as platelets, and that related proteins selective for the Type I enzyme are present. As yet, there have been no reports describing AKAPs for Type I PKAs. However, in a study performed on T lymphocytes, Skalhegg et al. (1994) demonstrated that stimulation with anti-CD3 antibodies led to the translocation of Type I PKA from the cytosol to the plasma membrane. Although the targeting mechanism has not yet been defined, these investigators postulate a possible interaction between the proline-rich regions near the N-terminus of the R_I-subunit and the SH3 domains of protein tyrosine kinases ($p56^{lck}$ or $p59^{fyn}$). Platelet membranes also contain several related tyrosine kinases, including $p60^{src}$, $p59^{fyn}$ and $p56/58^{lyn}$ (reviewed by Jackson et al., 1996).

5.1.2 What is the identity of the 65 kDa protein?

Photoaffinity labelling of human platelet proteins identified a 65 kDa labelled species, which was restricted to the membrane fraction. This 65 kDa photolabelled protein does not correspond to any known platelet cGMP binding protein and is very unlikely to be a proteolytic fragment of PKG since it was not immunoprecipitated with this enzyme. Moreover, photolabelling of PKG was inhibited by Mg^{2+}, whereas that of the 65 kDa protein was not. Since both cAMP and cGMP inhibited labelling of this protein, it is likely that this protein represents a novel membrane target for cyclic nucleotide action in platelets. Indeed, the similarity in size of this protein to the α-subunit (63 kDa) of the retinal rod CNG ion channel (Kaupp, 1991), and its location in

platelet membranes are consistent with the possibility that the 65 kDa protein may represent a subunit of a platelet cGMP-gated ion channel. Recently, a brief report has indicated that cDNA sequences more than 90% identical to the α-subunit of the rod photoreceptor channel can be amplified from MEG-01 cells and that cross-reactive protein can be detected immunologically in megakaryocytes and platelets (Surks et al., 1995). Since antibodies to the α-subunits of some CNG channels are available, it would be of interest to confirm the presence of a CNG channel protein in platelets by attempting to immunoprecipitate the 65 kDa photolabelled protein from platelet extracts.

CNG channel proteins are hetero-oligomers composed of at least two different subunits (α and β) (see section 4.2). Both α- and β-subunits of the retinal channel could be photolabelled by APT[^{32}P]cGMP (8-*p*-azidophenacylthio-cGMP), indicating that both subunits bind cGMP and may contribute to channel activation (Karpen and Brown, 1996). Attempts to purify the 65 kDa platelet membrane protein have revealed the presence of a 115 kDa photolabelled species in the same fractions (Haslam and Davidson, unpublished). It is possible that this larger protein may represent an additional subunit or component of a platelet CNG channel.

5.1.3 What is the value of this photolabelling technique?

This photolabelling method can be readily carried out using crude cell lysates and provides a simple and rapid means of studying cGMP-binding proteins. The

specificity of interaction between selected cGMP-binding proteins and various compounds can be established by monitoring the impact of the latter on the photolabelling of the former. Thus, selective inhibitors of PDE3 (e.g. trequinsin and cilostamide) were demonstrated to block photolabelling of PDE3 in platelet supernatant fraction, whereas inhibitors of PKA (Rp-cAMPS) or PKG (Rp-8-bromo-cGMPS) were without effect (see Chapter 3). Consequently, this photolabelling procedure permits rapid identification and comparison of specific inhibitors of platelet PDE3.

Despite the low efficiency of ^{32}P incorporation (about 0.1%), quantitative analysis of the effects of PDE3 inhibitors on the binding of [^{32}P]cGMP to PDE3 remained quite feasible (see Chapter 3). Compounds such as cilostamide, trequinsin, milrinone, lixazinone and siguazodan were all demonstrated to inhibit PDE3 photolabelling in a concentration-dependent manner. In general, the IC_{50} values for inhibition of photolabelling were comparable to the previously reported values for inhibition of enzyme activity, despite the variability in substrate concentration and extent of enzyme purification in the published studies. Although the concentrations of inhibitors required to stimulate cAMP accumulation in platelets (EC_{50}) were substantially higher than the concentrations necessary to inhibit photolabelling of PDE3 (IC_{50}), the rank order of effectiveness of these inhibitors in both situations remained the same. These discrepancies in IC_{50} and EC_{50} values probably relate to differences in the rates of entry of the individual inhibitors into the intact platelet. As a result, brief incubation periods may lead to a need for higher concentrations of some

compounds to induce increases in platelet cAMP or inhibition of platelet aggregation than for inhibition of enzyme activity in platelet extracts.

In addition, the general applicability of this photoaffinity labelling procedure was demonstrated by labelling rat tissues with [^{32}P]cGMP. Distinctive photolabelling patterns were observed and several novel cGMP-binding proteins were detected. Interestingly, the 110 kDa protein found in human platelets was replaced by a 115 kDa species in rat platelets. This larger protein was selectively inhibited by cilostamide and lixazinone, establishing its identity as the rat PDE3. A 175 kDa photolabelled species, which does not correspond to any known platelet cGMP-binding protein, was also detected in rat tissues, most notably in the spleen and liver. Due to probable but unknown differences in the efficiencies of photolabelling different proteins with [^{32}P]cGMP, an accurate measure of the relative amounts of different cGMP-binding proteins *within* individual tissues is not possible with this technique. However, comparison of the relative distributions of specific cGMP targets *between* different tissues is feasible, and may provide an indication of the relative importance of different actions of cGMP in different tissues. For example, photolabelling of the 80 kDa protein (PKG) was barely detectable in rat platelets, but was much more prominent in heart, aorta, uterus and lung tissues, suggesting a more important functional role in the latter tissues.

5.2 Molecular cloning of platelet PDE3

5.2.1 Identification of the platelet PDE3 isoenzyme

The platelet PDE3 enzyme was demonstrated to be a product of the PDE3A gene using an RT-PCR approach (see Chapter 4). Degenerate primers, based on published platelet peptide sequences, and purified platelet mRNA, were used to generate a partial clone that was identical in sequence to a segment (560-1146 amino acid) of the human cardiac enzyme cloned by Meacci et al. (1992). Although both the platelet and cardiac enzymes are clearly derived from the same gene, differences with respect to size, subcellular distribution and inhibitor sensitivities have been noted. The cytosolic distribution and smaller subunit size (110 kDa) of the platelet enzyme distinguishes it from the larger (126 kDa) membrane-associated cardiac enzyme. In addition, the PDE3 inhibitor, vesnarinone has been reported to inhibit the cardiac form of PDE3 to a greater extent than the platelet enzyme (Masuoka et al., 1993). Although a recently cloned 75 kDa placental PDE3A was found to be 100% identical in sequence to a portion of the cardiac enzyme, the generation of this shorter enzyme has been attributed to transcriptional initiation from a distinct site (Kasuya et al., 1995). Northern blot analysis and RNAse protection assays identified the presence of two transcripts of 4.4 kb and 7.6 kb in human placenta, coding for cytosolic and membrane-associated forms, respectively. Very recently, cDNA sequences corresponding to the N-terminus of the membrane-bound cardiac enzyme have been amplified from platelet mRNA (Cheung et al., 1996), though there is no direct evidence for expression of the intact cardiac enzyme in platelets. Determination of the

sizes of PDE3A mRNA in present platelets would help to clarify the relationship between the cardiac and platelet enzymes. However, attempts by us and others to analyze platelet PDE3A mRNA by northern blotting have been unsuccessful. Nevertheless, it remains possible that differential splicing or an alternate transcriptional initiation site could account for the observed differences between the platelet and cardiac forms of PDE3. These issues will be clarified when the full genomic sequence of human PDE3A becomes available.

5.2.2 Cloning and functional expression of the C-terminal half of platelet PDE3

Sequences corresponding to the C-terminal half of platelet PDE3 were cloned and expressed in a bacterial system (see Chapter 4). A previous study by Harrison et al. (1986) has demonstrated that the immunoprecipited enzyme retained activity. The anti-peptide antibody utilized in my studies was directed against the C-terminus of PDE3 and did not have a disruptive effect on PDE3 activity. Thus, comparisons of the enzyme kinetics and inhibitor sensitivities of immunoprecipitates of N-terminal truncation mutants with those of immunoprecipitates of full-length native enzyme were feasible using this technique. The K_m values for hydrolysis of cAMP and cGMP in this study, were lower for the N-terminal deletion mutants than for the intact enzyme. The effects of N-terminal deletion of the PDE3A gene product on enzyme kinetics reported in the literature, have been quite variable and sometimes contradictory (see Table 1). For example, Pillai et al. (1994) reported that truncation mutants of PDE3A expressed in yeast demonstrated *higher* K_m values for cAMP hydrolysis than the

expressed intact enzyme. In contrast, Kasuya et al. (1995) reported that the K_m for cAMP hydrolysis was *unchanged* for the baculovirus expressed placental enzyme, but that the K_m for cGMP hydrolysis was *increased* relative to the intact enzyme. Very recently, another group (Leroy et al., 1996) analysed two additional N-terminal deletion mutants of the cardiac enzyme, which were expressed in Sf9 cells. These mutants exhibited K_m values for cAMP hydrolysis that were similar to those of the 'intact' enzyme. Some of these variations in the reported kinetic values could be attributed to differences in the assay conditions (Mn^{2+} vs Mg^{2+}) and in the expression systems utilized by the different investigators. In the above studies, no attempt was made to isolate PDE3 from the other proteins present in the yeast and insect cells and it is therefore also possible that the observed variations in kinetic data reflect the modifying influence of contaminating cellular proteins. In contrast, the studies described in this thesis (Chapter 4), employed enzyme preparations purified by immunoprecipitation or nickel-resin column chromatography. Although truncation mutants purified by both techniques exhibited similar kinetic profiles, only the immunoprecipitation procedure permitted a direct comparison of the truncation mutants with platelet PDE3A. Since previous photolabelling studies established that *intact* PDE3A could be obtained from platelet extracts by immunoprecipitation (see Chapter 2), this study represents the first report directly comparing full-length non-proteolyzed PDE3A with various truncation mutants. A recent study by Cheung et al. (1996) reported that the K_m values for cAMP hydrolysis by PDE3A deletion mutants expressed in yeast cells, were identical to those obtained with purified platelet PDE3A.

Table 1 Comparison of the kinetic values of PDE3 deletion mutants

Enzyme	Amino acid sequence*	K_m (cAMP) (nM)	K_m (cGMP) (nM)	Reference
Platelet	full-length	398 ± 50	252 ± 16	Chapter 4
cGI-PDEΔ1	560-1141	182 ± 12	153 ± 12	
cGI-PDEΔ2	659-1141	131 ± 17	99 ± 16	
Cardiac clone	1-1141	150 ± 10	-	Pillai et al., 1994
ACGIΔ4	398-1141	460 ± 80	-	
ACGIΔ5	511-1141	630 ± 80	-	
ACGIΔ6	608-1141	560 ± 160	-	
Cardiac clone	1-1141	-	460 ± 220	Kasuya et al., 1995
Placental clone	484-1141	500	3270 ± 1750	
Cardiac clone	1-1141	150 ± 30	-	Leroy et al., 1996
pVL/Δ5	613-1141	120 ± 30	-	
Platelet	purified	240 ± 30	-	Cheung et al., 1996
PDE3AΔ1	665-1141	200 ± 20	-	
PDE3AΔ2	679-1141	230 ± 30	-	

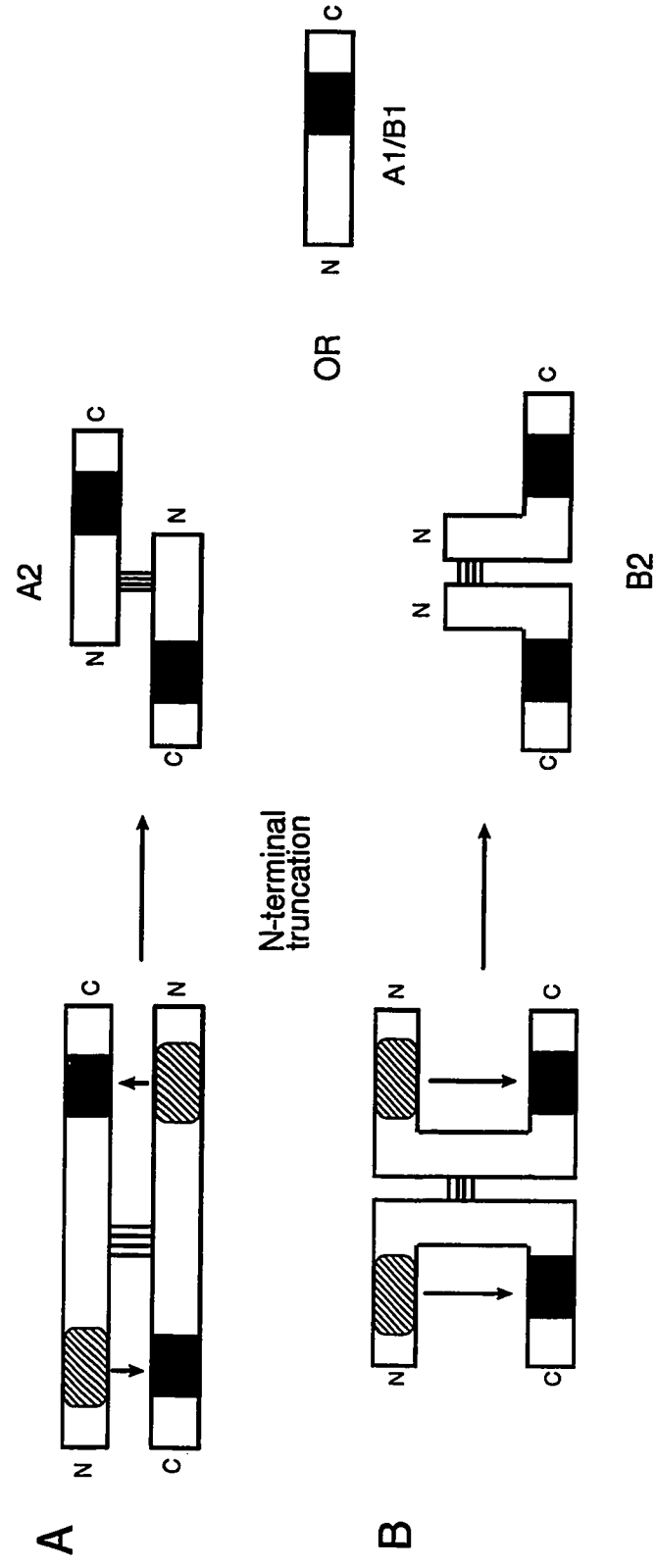
* Amino acid numbers were determined according to the cardiac sequence published by Meacci et al. (1992).

However, if these authors isolated proteolyzed forms of PDE3A, it would not be unexpected if they failed to detect changes in the K_m of the enzyme for cAMP. In the present study, the K_m values for *both* cAMP and cGMP hydrolysis were lower for the deletion mutants than for the intact native enzyme. This study is also the first to demonstrate that successive deletions of N-terminal sequences are accompanied not only by progressively lower K_m values, but also by a significantly increased V_{max} for cGMP relative to that for cAMP. Thus, the process of N-terminal deletion increases the activity of the enzyme for cGMP relative to cAMP, implying that N-terminal sequences directly or indirectly exert either a selective inhibitory effect on the hydrolysis of cGMP or a selective stimulatory effect on the hydrolysis of cAMP.

Two suggested models of PDE3, incorporating a proposed N-terminal 'modulatory' domain, are illustrated in figure 2. Model A postulates an inter-subunit mechanism in which the 'modulatory' domain of one subunit of the PDE3 homodimer affects the substrate specificity of the catalytic domain of the second subunit. In contrast, model B suggests that the modification of catalytic activity is derived from a region located within the same subunit. According to these models, deletion of the N-terminal 'modulatory' domain could generate either monomers (A1 or B1) or dimers (A2 or B2) exhibiting altered catalytic activity. Further studies will be needed to determine whether the expressed truncation mutants are in fact monomers or dimers; this may permit identification of the site of subunit interaction. The distinctive property of members of the PDE3 gene family is the ability of cGMP to inhibit

Figure 2 Suggested models of PDE3

Two models of PDE3 are proposed which incorporate an N-terminal 'modulatory' domain into the dimer structure. In model A, the modulatory domain of one subunit of the dimer acts on the catalytic domain of the second subunit (inter-subunit mechanism), whereas in model B, the modification of substrate specificity of the catalytic activity of PDE3 is derived from an N-terminal region located within the same subunit (intra-subunit mechanism). Deletion of the 'modulatory' domain could generate either monomers (A1 or B1) or dimers (A2 or B2) depending on the extent of the N-terminal truncation and the site of subunit interaction.


A
B
N-terminal truncation
A1/B1
OR
A2
B2
C
N

enzyme activity. It is the combination of a low K_m and low V_{max} for cGMP that is responsible for this property and thus for the physiological regulation of cAMP hydrolysis by cGMP in platelets. As a whole, my results suggest that it is N-terminal sequences in PDE3 that are partly responsible for the low cGMP hydrolysis (relative to cAMP) that are characteristic of the enzyme.

The sensitivities of the intact platelet enzyme and the N-terminal deletion mutants to the selective PDE3 inhibitor, lixazinone, were compared. The K_m of the intact platelet enzyme was increased by lixazinone, whereas the V_{max} remained unchanged, indicating that lixazinone is a competitive inhibitor. In contrast, the N-terminal deletion mutant demonstrated a mixed competitive and uncompetitive inhibition by lixazinone. The inhibitory potency of lixazinone may stem from the molecular interaction between the compound and a bulk-tolerant lipophilic binding site within the enzyme (Venuti et al., 1988). Since inhibition by this compound changes from predominantly competitive for the intact enzyme to mixed in the deletion mutant, it is possible that removal of the N-terminus provides the inhibitor with access to an additional binding site.

A characteristic feature of members of the PDE3 gene family is the presence of a 44 amino acid insertion located within the catalytic domain. I and others (Manganiello et al., 1995) initially thought that this insert, which bears no similarity to other sequences currently in the protein database, might contribute to the unique

properties of the PDE3 gene family. Specific deletion of these 44 amino acids from the catalytic domain was accomplished using a PCR approach (see Chapter 4). Although enzyme activity was abolished by the deletion of this insert, a limited ability to bind cGMP, as detected by photolabelling, was retained. Structural analysis (Garnier et al., 1978) predicts β-turns at the C and N-termini of the 44 amino acid inserts in both the PDE3A and PDE3B gene products. Site-directed mutagenesis of these two putative β-turns, as well as of several other conserved residues, was therefore undertaken to identify functionally important amino acid residues in the PDE3A insert. Although disruption of a cluster of negatively charged residues had no major effects on hydrolytic activity, site-directed mutagenesis of the putative N-terminal or C-terminal β-turns markedly reduced enzyme activity. This study is the first reported attempt to define a functional role for the 44 amino acid insert by site-directed and deletion mutagenesis. The results indicate that the insert is required to preserve an effective catalytic domain structure in PDE3, though not in other PDE gene families. This could relate to the positioning of specific residues in the catalytic centre. Alternatively, its role could be to assist in the proper folding of the catalytic region of the PDE3 enzyme. Since the mutant lacking the 44 amino acid insert (cGI-PDEΔ1Δi) was devoid of enzyme activity, it would be interesting to attempt to restore partial or full enzyme activity by reinserting different lengths of flexible neutral amino acid sequence (e.g. Gly_4 or Gly_8). Moreover, within the 44 amino acid insert there still remain several additional unmutated residues (N8, H10, Y29, S32, K33 and D39) that are conserved in both the PDE3A and PDE3B genes which could have functional

importance. Mutagenesis of these residues alone or in combination could also prove valuable.

5.3 Actions of cGMP in platelets

Very early studies on cGMP in platelets suggested a possible role in the stimulation of platelet aggregation (see section 1.4.2). However, it is now quite clear that increases in cGMP function to inhibit platelet aggregation. Indeed compounds such as SNP, SIN-1 and NO, which stimulate cGMP synthesis, are known to inhibit platelet activation (Haslam et al, 1980; Mellion et al, 1981). Moreover, membrane-permeant analogues of cGMP have been demonstrated to elicit similar inhibitory effects (Butt et al., 1990; 1992).

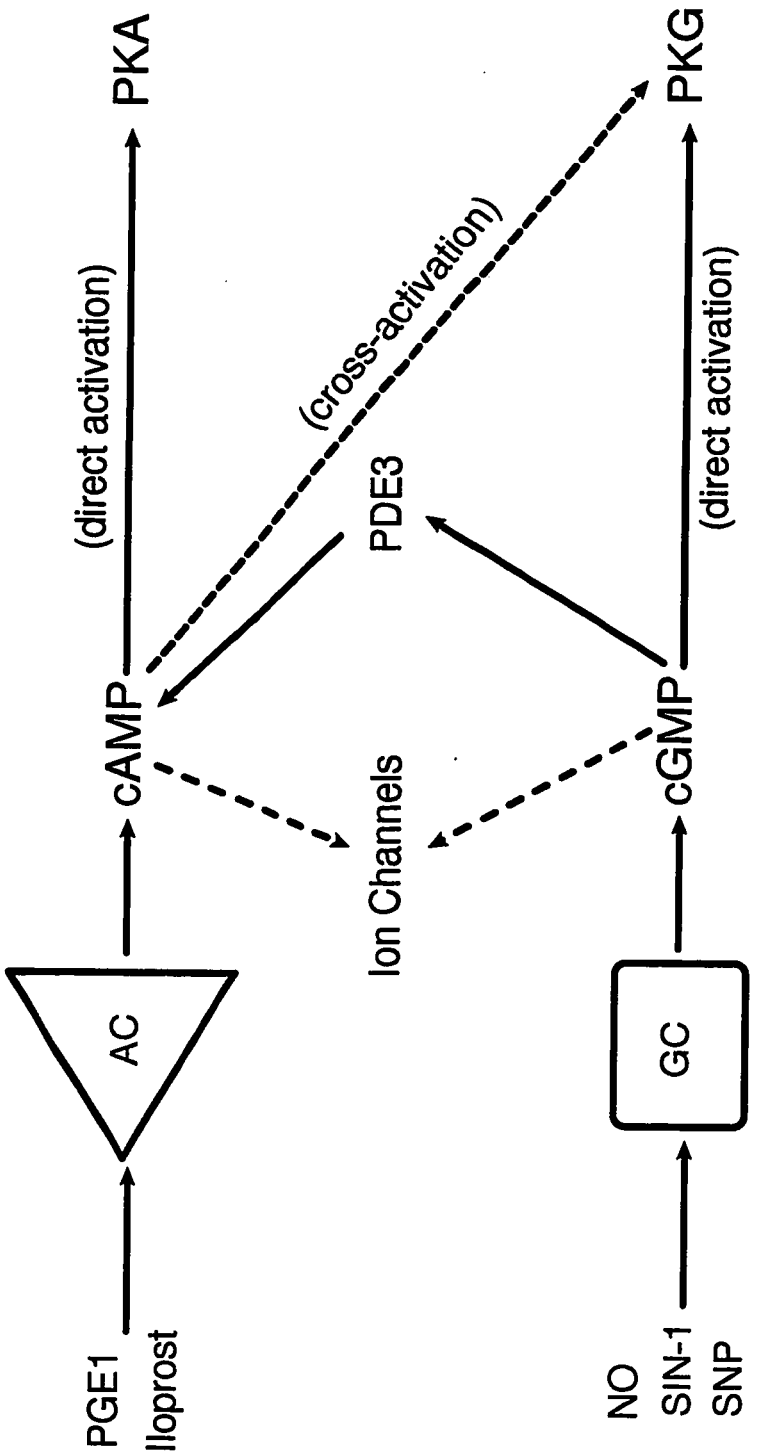
cGMP is able to exert effects through several distinct targets including protein kinases, phosphodiesterases and ion channels. It is well established now that PKG has a physiological role as a mediator of the action of cGMP in human platelets. Activation of PKG by cGMP has been demonstrated to stimulate phosphorylation of specific proteins associated with the inhibition of platelet activation (Haslam et al., 1980; Waldman et al., 1986). Some investigators have contended that the role of cGMP in the inhibition of platelet activation is solely mediated by PKG, without consideration of the possible contributions made by other proteins (Walter, 1989; Moncada et al., 1990; Moro et al., 1996). Since cGMP also directly inhibits PDE3, this enzyme is a likely site of cGMP action. Indeed, the first evidence that PDE3 is an

important mediator of cGMP action came from studies with rabbit platelets (Maurice and Haslam, 1990a; Bowen and Haslam, 1991). These investigators demonstrated that increases in cGMP resulting from addition of SNP or SIN-1 to platelets, inhibited PDE3 activity and enhanced both cAMP accumulation and the inhibition of platelet function by activators of AC (see section 4.3.2 and figure 3). This mechanism of cGMP action has since been demonstrated in human platelets (Fisch et al., 1995) and in other tissues including aortic smooth muscle (Maurice and Haslam, 1991). The model depicted in figure 3 illustrates the ability of PDE3 to mediate effects of cGMP on platelet function. Thus, nitrovasodilator-induced increases in cGMP can act synergistically to increase platelet cAMP and inhibit platelet activation, indicating that PDE3 can make an important contribution to the action of these compounds. This cAMP could activate both PKA and PKG (figure 3). Indeed, my observation that cAMP can enhance photolabelling of PKG (see Chapter 2), suggests a novel mechanism of co-operative interaction between cAMP and cGMP. It follows that the proposed role of PDE3 in the action of cGMP does not necessarily conflict with the evidence that activation of PKG plays a major role in the effects of nitrovasodilators on platelet function (Walter, 1989; Halbrugge and Walter, 1993; Moro et al., 1996).

In the past, it was generally accepted that cAMP and cGMP represented components of important but independent signalling pathways. Indeed, different cellular responses were reported to be solely mediated by one or the other compound acting as a second messenger. In certain tissues, however, the ability of cyclic

Figure 3 Scheme showing cGMP actions in platelets

The potential targets of cGMP in platelets include PKG, PDE3 and ion channels. Increases in cGMP resulting from the actions of nitrovasodilators could activate PKG directly by binding to this enzyme or indirectly by inhibiting PDE3. The latter possibility enhances cAMP accumulation which could then activate PKA and cross-activate PKG.


PGE1
Iloprost
AC
cAMP
PKA
(direct activation)
(cross-activation)
PDE3
Ion Channels
NO
SIN-1
SNP
GC
cGMP
PKG
(direct activation)

nucleotides to cross-activate protein kinases has been described (see section 4.1.2) and provides an additional mechanism for regulation of cellular function by these compounds. Thus, when PKA and PKG are present within the same cell, increases in either cAMP or cGMP could, in principle, lead to activation of both protein kinases. Moreover, tissues expressing only one kinase form (PKA or PKG) could be regulated by increases in either cyclic nucleotide. Since autophosphorylation can decrease the concentration of cAMP required for PKG activation (K_a) (Landgraf et al., 1986), it would not be unusual for cAMP to function as an important mediator of PKG activation in certain tissues. Indeed, the increases in cAMP resulting from the inhibitory action of cGMP on PDE3 could have an additional effect on PKG activation. This cAMP accumulation may represent an amplification mechanism enhancing the activation of PKG by cGMP (see figure 3). Consequently, the previously held view that cAMP and cGMP act independently of each other must give way to a more complex model in which cAMP and cGMP function in concert to mediate cellular responses.

In conclusion, cGMP is able to exert important cellular effects by acting not only directly on PKG, but also via the inhibition of PDE3. Although the presence of CNG ion channels has not as yet been demonstrated in platelets, the identification of a 65 kDa photolabelled species lends support to their possible presence in platelets, and suggests an additional target for cGMP action. Thus, my studies have enhanced our understanding of the multiple modes of action of cGMP and have defined some of the important functional and structural domains within a major target of cGMP (PDE3).

CHAPTER 6

REFERENCES

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.

Alvarez, R., A. Taylor, J.J. Fazzari and J.R. Jacobs (1981). Regulation of cyclic AMP metabolism in human platelets. Sequential activation of adenylate cyclase and cyclic AMP phosphodiesterase by prostaglandins. Mol. Pharmacol. **20**: 302-309.

Antonoff, R. S. and J.J. Ferguson Jr. (1978) Direct photo-affinity labeling with cyclic nucleotides: action spectra. Photochem. Photobiol. **27**: 499-501.

Armstead, W. (1995). Role of nitric oxide and cAMP in prostaglandin-induced pial arterial vasodilation. Am. J. Physiol. **268**: H1436-H1440.

Arshavsky, V.Y., C.L. Dumke and M.D. Bownds (1992). Noncatalytic cGMP-binding sites of amphibian rod cGMP phosphodiesterase control interaction with its inhibitory gamma-subunits. A putative regulatory mechanism of the rod photoresponse. J. Biol. Chem. **267**: 24501-24507.

Ball, G., G.G. Brereton, M. Fulwood, D.M. Ireland and P. Yates (1970). Effects of prostaglandin E1 alone and incombination with theophylline or aspirin on collagen-induced platelet aggregation and on platelet nucleotides including adenosine 3′:5′-cyclic monophosphate. Biochem. J. **120**: 709-718.

Barrington, W.W., K.A. Jacobson, A.J. Hutchison, M. Williams and G.L. Stiles (1989). Identification of the A2 adenosine receptor binding subunit by photoaffinity crosslinking. Proc. Natl. Acad. Sci. USA **86**: 6572-6576.

Beavo, J.A. (1988). Multiple isozymes of cyclic nucleotide phosphodiesterase. Adv. Sec. Mess. Phospho. Res. **22**: 1-38.

Beavo, J.A. (1995). Cyclic nucleotide phosphodiesterases: functional implications of multiple isoforms. Physiol. Rev. **75**: 725-748.

Beavo, J.A., J.G. Hardman and E.W. Sutherland (1970). Hydrolysis of cyclic guanosine and adenosine 3′,5′-monophosphates by rat and bovine tissues. J. Biol. Chem. **245**: 5649-5655.

Beebe, S. (1994). The cAMP-dependent protein kinases and cAMP signal transduction. Sem. Cancer Biol. **5**: 285-294.

Beebe, S., O. Oyen, M. Sandberg, A. Froysa, V. Hansson and T. Jahnsen (1990). Molecular cloning of a tissue-specific protein kinase (Cγ) from human testis

representing a third isoform for the catalytic subunit of cAMP-dependent protein kinase. Mol. Endocrinol. **5**: 465-475.

Beebe, S., P. Salmonsky, T. Jahnsen and Y. Li (1992). The Cγ-subunit is a unique isozyme of the cAMP-dependent protein kinase. J. Biol. Chem. **267**: 25505-25512.

Bentley, J.K., A. Kadlecek, C.H. Sherbert, D. Seger, W.K. Sonnenburg, H. Charbonneau, J.P. Novack and J.A. Beavo (1992). Molecular cloning of cDNA encoding a "63"-kDa calmodulin-stimulated phosphodiesterase from bovine brain. J. Biol. Chem. **267**: 18676-18682.

Berridge, M. (1993). Inositol trisphosphate and calcium signalling. Nature **361**: 315-325.

Berridge, M.J. (1995). Capacitative calcium entry. Biochem. J. **312**: 1-11.

Biel, M., X. Zong, M. Distler, E. Bosse, N. Klugbauer, M. Murakami, V. Flockerzi and F. Hofmann (1994). Another member of the cyclic nucleotide-gated channel family, expressed in testis, kidney, and heart. Proc. Natl. Acad. Sci. USA **91**: 3505-3509.

Blockmans, D., H. Deckmyn and J. Vermylen (1995). Platelet activation. Blood Rev. **9**: 143-156.

Bloom, T.J. and J.A. Beavo (1994). Identification of type VII PDE in HUT78 T-lymphocyte cells (Abstract). FASEB J. **8**: A372.

Bowen, R. and R.J. Haslam (1991). Effects of nitrovasodilators on platelet cyclic nucleotide levels in rabbit blood; role for cyclic AMP in synergistic inhibition of platelet function by SIN-1 and prostaglandin E_1. J. Card. Pharmacol. **17**: 424-433.

Boyes, S. and E.G. Loten (1988). Purification of an insulin-sensitive cyclic AMP phosphodiesterase from rat liver. Eur. J. Biochem. **174**: 303-309.

Brass, L.F., J.A. Hoxie and D.R. Manning (1993). Signaling through G proteins and G protein-coupled receptors during platelet activation. Thromb. Haemost. **70**: 217-223.

Bredt, D.S. and S.H. Snyder (1990). Isolation of nitric oxide synthetase, a calmodulin-requiring enzyme. Proc. Natl. Acad. Sci. USA **87**: 682-685.

Brown, R.L., W.V. Gerber and J.W. Karpen (1993). Specific labeling and permanent activation of the retinal rod cGMP activated channel by the photoaffinity analog 8-*p* azidophenacylthio-cGMP. Proc. Natl. Acad. Sci. USA **90**: 5369-5373.

Brown, R.L., R. Gramling, R.J. Bert and J.W. Karpen (1995). Cyclic GMP contact points within the 63 kDa subunit and a 240 kDa associated protein of retinal rod cGMP-activated channels. Biochemistry **34**: 8365-8370.

Brune, B., F. von Appen and V. Ullrich (1994). Receptor occupancy regulates Ca^{2+} entry and intracellular Ca^{2+} redistribution in activated human platelets. Biochem. J. **304 (Pt 3)**: 993-999.

Buechler, W., M. Nakene and F. Murad (1991). Expression of soluble guanylate cyclase activity requires both enzyme subunits. Biochem. Biophys. Res. Commun. **174**: 351-357.

Burns, F. and N. Pyne (1992a). Interaction of the catalytic subunit of protein kinase A with the lung type V cyclic GMP phosphodiesterase: modulation of non-catalytic binding sites. Biochem. Biophys. Res. Commun. **189**: 1389-1396.

Burns, F., I.W. Rodger and N.J. Pyne (1992b). The catalytic subunit of protein kinase A triggers activation of the type V cyclic GMP-specific phosphodiesterase from guinea-pig lung. Biochem. J. **283**: 487-491.

Butt, E., K. Abel, M. Krieger, D. Palm, V. Hoppe, J. Hoppe and U. Walter (1994). cAMP- and cGMP-dependent protein kinase phosphorylation sites of the focal adhesion vasodilator-stimulated phosphoprotein (VASP) in vitro and in intact human platelets. J. Biol. Chem. **269**: 14509-14517.

Butt, E., J. Geiger, T. Jarchau, S.M. Lohmann and U. Walter (1993). The cGMP-dependent protein kinase - gene, protein and function. Neurochem. Res. **18**:27-42.

Butt, E., C. Nolte, S. Schulz, J. Beltman, J.A. Beavo, B. Jastorff and U. Walter (1992). Analysis of the functional role of cGMP-dependent protein kinase in intact human platelets using a specific activator 8-para-chlorophenylthio-cGMP. Biochem. Pharmacol. **43**: 2591-2600.

Butt, E., M. van Bemmelen, L. Fischer, U. Walter and B. Jastorff (1990). Inhibition of cGMP-dependent protein kinase by (Rp)-guanosine 3′,5′-monophosphorothioates. FEBS Lett. **263**: 47-50.

Camps, M., A. Carozzi, P. Schnabel, A. Scheer, P.J. Parker and P. Gierschik (1992). Isozyme-selective stimulation of phospholipase C-beta 2 by G protein βγ-subunits. Nature **360**: 684-686.

Cavallini, L., M. Coassin, A. Borean and A. Alexandre (1996). Prostacyclin and sodium nitroprusside inhibit the activity of the platelet inositol 1,4,5-trisphosphate receptor and promote its phosphorylation. J. Biol. Chem. **271**: 5545-5551.

Charbonneau, H., S. Kumar, J.P. Novack, D.K. Blumenthal, P.R. Griffin, J. Shabanowitz, D.F. Hunt, J.A. Beavo and K.A. Walsh (1991). Evidence for domain organization within the 61-kDa calmodulin-dependent cyclic nucleotide phosphodiesterase from bovine brain. Biochemistry **30**: 7931-7940.

Charbonneau, H., R.K. Prusti, H. LeTrong, 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. USA **87**: 288-292.

Cheung, P., H. Xu, F. Ghazaleh, G. Livi and R.W. Colman (1996). Human platelet cGI-PDE: Expression in yeast and localization of the catalytic domain by deletion mutagenesis. Blood **4**: 1321-1329.

Chiang, T.M., D.S. N and A.H. Kang (1976). Effect of cyclic 3′,5′-guanosine monophosphate on human platelet function. J. Lab. Clin. Med. **88**: 215-221.

Chinkers, M. and D.L. Garbers (1989). The protein kinase domain of the ANP receptor is required for signaling. Science **245**: 1392-1394.

Clemetson, K.J. (1995). Platelet activation: signal transduction via membrane receptors. Thromb. Haemost. **74**: 111-116.

Cole, B., G.A. Robison and R.C. Hartmann (1971). Studies on the role of cyclic AMP in platelet function. Ann. NY Acad. Sci. **185**: 477-487.

Connolly, A.J., H. Ishihara, M.L. Kahn, R.V. Farese Jr. and S.R. Coughlin (1996). Role of the thrombin receptor in development and evidence for a second receptor. Nature **381**: 516-519.

Conti, M., G. Nemoz, C. Sette and E. Vicini (1995). Recent progress in understanding the hormonal regulation of phosphodiesterases. Endocr. Rev. **16**: 370-389.

Conti, M. and J. Swinnen (1990). Structure and Function of the Rolipram-sensitive, Low-K_m cyclic AMP phosphodiesterases: A family of highly related enzymes. in Cyclic nucleotide phosphodiesterases:structure, regulation and drug action. J. Beavo and M. Houslay (eds). New York, John Wiley & sons. Vol. 2. p. 243-266.

Coquil, J.F., D.J. Franks, J.N. Wells, M. Dupuis and P. Hamet (1980). Characteristics of a new binding protein distinct from the kinase for guanosine 3′:5′-monophosphate in rat platelets. Biochim. Biophys. Acta **631**: 148-165.

Cote, R.H., M.D. Bownds and V.Y. Arshavsky (1994). cGMP binding sites on photoreceptor phosphodiesterase: role in feedback regulation of visual transduction. Proc. Natl. Acad. Sci. USA **91**: 4845-4849.

Culty, M., M.M.L. Davidson and R.J. Haslam (1988). Effects of guanosine 5′-[γ-thio]triphosphate and thrombin on the phosphoinositide metabolism of electropermeabilized platelets. Eur. J. Biochem. **171**: 523-533.

Currie, M.G., D.M. Geller, B.R. Cole, J.G. Boylan, W. YuSheng, S.W. Holmberg and P. Needleman (1983). Bioactive cardiac substances: potent vasorelaxant activity in mammalian atria. Science **221**: 71-73.

Daniel, J.L., I.R. Molish and H. Holmsen (1981). Myosin phosphorylation in intact platelets. J. Biol. Chem. **256**: 7510-7514.

Daniel, J.L., I.R. Molish, 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.

De Graff, J.C., J.D. Banga, S. Moncada, R.M.J. Palmer, P.G. de Groot and J.J. Sixma (1992). Nitric oxide functions as an inhibitor of platelet adhesion under flow conditions. Circulation **85**: 2284-2290.

Degerman, E., P. Belfrage, A.H. Newman, K.C. Rice and V.C. Manganiello (1987). Purification of the putative hormone-sensitive cyclic AMP phosphodiesterase from rat adipose tissue using a derivative of cilostamide as a novel affinity ligand. J. Biol. Chem. **262**: 5797-5807.

Degerman, E., M. Moos Jr., A. Rascon, V. Vasta, E. Meacci, C.J. Smith, S. Lindgren, K.E. Andersson, P. Belfrage and V.C. Manganiello (1994). Single-step affinity purification, partial structure and properties of human platelet cGMP inhibited cAMP phosphodiesterase. Biochim. Biophys. Acta. **1205**: 189-198.

Degerman, E., C.J. Smith, H. Tornqvist, V. Vasta, P. Belfrage and V.C. Manganiello (1990). Evidence that insulin and isoprenaline activate the cGMP-inhibited low-K_m cAMP phosphodiesterase in rat fat cells by phosphorylation. Proc. Natl. Acad. Sci. USA **87**: 533-537.

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. USA **85**: 2424-2428.

Dickinson, N., E.K. Jang and R.J. Haslam (1996). cGMP regulates platelet cAMP levels through effects on both cGMP-inhibited and cGMP-stimulated phosphodiesterases. FASEB J. **10**: A2159.

Distler, M., M. Biel, V. Flockerzi and F. Hofmann (1994). Expression of cyclic nucleotide-gated cation channels in non-sensory tissues and cells. Neuropharmacology **33**: 1275-1282.

Dohlman, H., J. Thorner, M. Caron and R. Lefkowitz (1991). Model systems for the study of seven-transmembrane-segment receptors. Annu. Rev. Biochem. **60**: 653-688.

Doni, M., L. Cavallini and A. Alexandre (1994). Ca^{2+} influx in platelets: activation by thrombin and by the depletion of the stores. Effect of cyclic nucleotides. Biochem. J. **303 (Pt 2)**: 599-605.

Drewett, J.G. and D.L. Garbers (1994). The family of guanylyl cyclase receptors and their ligands. Endocr. Rev. **15**: 135-162.

Eckly, A. and C. Lugnier (1994). Role of phosphodiesterases III and IV in the modulation of vascular cyclic AMP content by the NO/cyclic GMP pathway. Br. J. Pharmacol. **113**: 445-450.

Erneux, C., D. Couchie, J. Dumont, J. Baraniak and W. Stec (1981). Specificity of cyclic GMP activation of multi-substrate cyclic nucleotide phosphodiesterase from rat liver. Eur. J. Biochem. **115**: 503-510.

Falcon, C., G. Pfliegler, H. Deckmyn and J. Vermylen (1988). The platelet insulin receptor: detection, partial characterization, and search for a function. Biochem. Biophys. Res. Commun. **157**: 1190-1196.

Finn, J., M. Grunwald and K.-W. Yau (1996). Cyclic nucleotide-gated ion channels: An extended family with diverse functions. Annu. Rev. Physiol. **58**: 395-426.

Fisch, A., J. Michael-Hepp, J. Meyer and H. Darius (1995). Synergistic interaction of adenylate cyclase activators and nitric oxide donor SIN-1 on platelet cyclic AMP. Eur. J. Pharmacol. **289**: 455-461.

Fischmeister, R. and H. Hartzell (1990). Regulation of calcium current by low-K_m cyclic AMP phosphodiesterases in cardiac cells. Mol. Pharmacol. **38**: 426-433.

Fiscus, R., H. Hao, X. Wang, W. Arden and J. Diana (1994). Nitroglycerine (exogenous nitric oxide) substitutes for endothelium-derived nitric oxide in potentiating vasorelaxations and cyclic AMP elevations induced by Calcitonin Gene-related Peptide (CGRP) in rat aorta. Neuropeptides **26**: 133-144.

Fitzgerald, L.A., B. Steiner, S.C. Rall Jr., S.S. Lo and D.R. Phillips (1987). Protein sequence of endothelial glycoprotein IIIa derived from a cDNA clone. Identity with platelet glycoprotein IIIa and similarity to "integrin". J. Biol. Chem. **262**: 3936-3939.

Florio, V.A., W.K. Sonnenburg, R. Johnson, K.S. Kwak, G.S. Jensen, K.A. Walsh and J.A. Beavo (1994). Phosphorylation of the 61-kDa calmodulin-stimulated cyclic nucleotide phosphodiesterase at serine 120 reduces its affinity for calmodulin. Biochemistry **33**: 8948-8954.

Forte, L., P.K. Thorne, S.L. Eber, W.J. Krause, R.H. Freeman, S.H. Francis and J.D. Corbin (1992). Stimulation of intestinal Cl- transport by heat-stable enterotoxin: activation of cAMP-dependent protein kinase by cGMP. Am. J. Physiol. **263**: C607-C615.

Fox, J.E.B. (1993). The platelet cytoskeleton. Thromb. Haemost. **70**: 884-893.

Francis, S.H., J.L. Colbran, L.M. McAllister-Lucas and J.D. Corbin (1994). Cyclic nucleotide phosphodiesterases are zinc hydrolyses as indicated by conserved zinc-binding motifs, specific zinc binding and zinc supported catalysis. (Abstract). FASEB J. **8**: A371.

Francis, S.H. and J.D. Corbin (1994). Progress in understanding the mechanism and function of cyclic GMP-dependent protein kinase. Adv. Pharmacol. **26**: 115-170.

Francis, S.H., T.M. Lincoln and J.D. Corbin (1980). Characterization of a novel cGMP binding protein from rat lung. J. Biol. Chem. **255**: 620-626.

Francis, S.H., B.D. Noblett, B.W. Todd, J.D. Wells and J.D. Corbin (1988). Relaxation of vascular and tracheal smooth muscle by cyclic nucleotide analogs that preferentially activate purified cGMP-dependent protein kinase. Mol. Pharmacol. **34**: 506-517.

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. J. Beavo and M.D. Houslay (eds). New York, John Wiley & Sons. Vol. 2. p. 117-140.

Frins, S., W. Bonigk, G. Muller, R. Kellner and K.-W. Koch (1996). Functional characterization of a guanylyl cyclase-activating protein from vertebrate rods. J. Biol. Chem. **271**: 8022-8027.

Fulle, H.J., R. Vassar, D.C. Foster, R.B. Yang, R. Axel and D.L. Garbers (1995). A receptor guanylyl cyclase expressed specifically in olfactory sensory neurons. Proc. Natl. Acad. Sci. USA **92**: 3571-3575.

Garnier, J., Osguthorpe, D.J. and B. Robson. (1978) Analysis of the accuracy and implications of simple methods for predicting the secondary structure of globular proteins. J. Mol. Biol. **120**: 97-120.

Geiger, J., C. Nolt, E. Butt, S.O. Sage and W. U (1992). Role of cGMP and cGMP-dependent protein kinase in nitrovasodilator inhibition of agonist-evoked calcium elevation in human platelets. Proc. Natl. Acad. Sci. USA **89**: 1031-1035.

Gerzer, R., E. Bohme, F. Hofmann and G. Schultz (1981). Soluble guanylate cyclase purified from bovine lung contains heme and copper. FEBS Lett. **132**: 71-74.

Ghazaleh, F., G. Omburo and C. R (1996). Evidence for the presence of essential histidine and cysteine residues in platelet cGMP-inhibited phosphodiesterase. Biochem. J. **317**: 495-501.

Gillespie, P.G. and J.A. Beavo (1989). Inhibition and stimulation of photoreceptor phosphodiesterases by dipyridamole and M&B 22,948. Mol. Pharmacol. **36**: 773-781.

Giuili, G., U. Scholl, F. Bulle and G. Guellaen (1992). Molecular cloning of the cDNAs coding for the two subunits of soluble guanylyl cyclase from human brain. FEBS Lett. **304**: 83-88.

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. USA **85**: 9071-9075.

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. Thromb. Res. **59**: 105-119.

Groppi, V.E., Steinberg, F., Kaslow, H.R., Walker, N. and Bourne, H.R. (1983) Identification by direct photo-affinity labeling of an altered phosphodiesterase in a mutant S49 lymphoma cell. **258**: 9717-9723.

Grunberg, B., E. Negrescu and W. Siess (1995). Synergistic phosphorylation of platelet rap 1b by SIN-1 and iloprost. Eur. J. Pharmacol. **288**: 329-333.

Haffner, C., T. Jarchau, M. Reinhard, J. Hoppe, S.M. Lohmann and U. Walter (1995). Molecular cloning, structural analysis and functional expression of the proline-rich focal adhesion and microfilament-associated protein VASP. EMBO J. **14**: 19-27.

Hallbrugge, 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 cAMP-elevating vasodilators. J. Biol. Chem. **265**: 3088-3093.

Hallbrugge, M. and U. Walter (1989). Purification of vasodilator-regulated phosphoprotein from human platelets. Eur. J. Biochem. **185**: 41-50.

Hallbrugge, M. and U. Walter (1993). The regulation of platelet function by protein kinases. in Protein kinases in blood cell function. C.-K. Huang and R.I. Sha'afi (eds). Boca Raton, CRC Press p. 245-298.

Hamet, P., J.F. Coquil, S. Bousseau-Lafortune, D.J. Franks and J. Tremblay (1984). Cyclic GMP binding and phosphodiesterase: implication for platelet function. Adv. Cyc. Nuc. Pro. Phosphor. Res. **16**: 119-136.

Hansen, R.S. and J.A. Beavo (1982). Purification of two calcium/calmodulin-dependent forms of cyclic nucleotide phosphodiesterase by using conformation-specific monoclonal antibody chromatography. Proc. Natl. Acad. Sci. USA **79**: 2788-2792.

Harrison, S.A., D.H. Reifsnyder, B. Gallis, G.G. Cadd and J.A. Beavo (1986). Isolation and characterization of bovine cardiac muscle cGMP-inhibited phosphodiesterase: a receptor for new cardiotonic drugs. Mol. Pharmacol. **29**: 506-14.

Harteneck, C., D. Koesling, A. Soling, G. Schultz and E. Bohme (1990). Expression of soluble guanylyl cyclase. Catalytic activity requires two enzyme subunits. FEBS Lett. **272**: 221-223.

Harteneck, C., B. Wedel, D. Koesling, J. Malkewitz, E. Bohme and G. Schultz (1991). Molecular cloning and expression of a new alpha-subunit of soluble guanylyl cyclase. Interchangeability of the alpha-subunits of the enzyme. FEBS Lett. **292**: 217-222.

Hartzell, H. and R. Fischmeister (1986). Opposite effects of cyclic GMP and cyclic AMP and Ca current in single heart cells. Nature **323**: 273-275.

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 (1977). Role of cyclic AMP in platelet function: evidence from inhibition of adenylate cyclase in intact platelets by adenosine analogues. Thromb. Haemost. **38**: 6.

Haslam, R.J., J.A. Lynham and J.E.B. Fox (1979). Effects of collagen, ionophore A23187 and prostaglandin E1 on the phosphorylation of specific proteins in blood platelets. Biochem. J. **178**: 397-406.

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.

A. Rotman, F. Meyer, C. Gitler and A. Silberberg (eds). Chichester, Wiley. p. 213-231.

Hayashi, F., G.Y. Lin, H. Matsumoto and A. Yamazaki (1991). Phosphatidylinositol-stimulated phosphorylation of an inhibitory subunit of cGMP phosphodiesterase in vertebrate rod photoreceptors. Proc. Natl. Acad. Sci. USA **88**: 4333-4337.

Haynes, J., D. Killilea, Peterson and W. Thompson (1996). Erythro-9-(2-hydroxy-3-nonyl)adenine inhibits cyclic-3′,5′-guanosine monophosphate-stimulated phosphodiesterase to reverse hypoxic pulmonary vasoconstriction in the perfused rat lung. J. Pharmacol. Exp. Ther. **276**: 752-757.

Heemskerk, J.W.M. and S.O. Sage (1994). Calcium signalling in platelets and other cells. Platelets **5**: 295-316.

Heemskerk, J.W.M., P. Vis, M.A.H. Feijge, J. Hoyland, W.T. Mason and S.O. Sage (1993). Roles of phospholipase C and Ca^{2+}-ATPase in calcium responses of single, fibrinogen bound platelets. J. Biol. Chem. **268**: 356-363.

Hempel, A., T. Noll, A. Muhs and H. Piper (1996). Functional antagonism between cAMP and cGMP on permeability of coronary endothelial monolayers. Am. J. Physiol. **270**: H1264-H1271.

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.

Hillery, C.A., S.S. Smyth and L.V. Parise (1991). Phosphorylation of human platelet glycoprotein IIIa (GPIIIa). Dissociation from fibrinogen receptor activation and phosphorylation of GPIIIa *in vitro*. J. Biol. Chem. **266**: 14663-14669.

Hirata, M., Y. Hayashi, F. Ushikubi, Y. Yokota, R. Kageyama, S. Nakanishi and S. Narumiya (1991). Cloning and expression of cDNA for a human thromboxane A2 receptor. Nature **349**: 617-620.

Hirata, T., U. Fumitaka, A. Kakizuka, M. Okuma and S. Narumiya (1996). Two thromboxane A2 receptor isoforms in human platelets. J. Clin. Invest. **97**: 949-956.

Ho, H.C., E. Wirch, F.C. Stevens and J.H. Wang (1977). Purification of a Ca^{2+}-activatable cyclic nucleotide phosphodiesterase from bovine heart by specific interaction with its Ca^{2+}-dependent modulator protein. J. Biol. Chem. **252**: 43-50.

Hofmann, F., Gensheimer, H.P., Landgraf, W., Hullin, R. and B. Jastorff. (1985) Diastereomers of adenosine 3′,5′-monothionophosphate (cAMP[S]) antagonize the

activation of cGMP-dependent protein kinase. Eur. J. Biochem. **150**: 85-88.

Hofmann, F., W. Dostmann, A. Keilbach, W. Landgra and P. Ruth (1992). Structure and physiological role of cGMP-dependent protein kinase. Biochim. Biophys. Acta **1135**: 51-60.

Horstrup, K., B. Jablonka, P. Honig-Liedl, M. Just, K. Koshsiek and U. Walter (1994). Phosphorylation of focal adhesion vasodilator-stimulated phosphoprotein at Ser157 in intact human platelets correlates with fibrinogen receptor inhibition. Eur. J. Biochem. **225**: 21-27.

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.

Humbert, P., F. Niroomand, G. Fischer, B. Mayer, D. Koesling, K. Hinsch, H. Gausepohl, R. Frank, G. Schultz and E. Bohme (1990). Purification of soluble guanylyl cyclase from bovine lung by a new immunoaffinity chromatographic method. Eur. J. Biochem. **190**: 273-278.

Huttemann, E., D. Ukena, V. Lenschow and U. Schwabe (1984). Ra adenosine receptors in human platelets. Characterization by 5'-N-ethylcarboxamido[3H]adenosine binding in relation to adenylate cyclase activity. Naunyn. Schmiedebergs Arch. Pharmacol. **325**: 226-233.

Ichimura, M. and H. Kase (1993). A new cyclic nucleotide phosphodiesterase isozyme expressed in the T-lymphocyte cell lines. Biochem. Biophys. Res. Commun. **193**: 985-990.

Ignarro, L.J. (1989). Heme-dependent activation of soluble guanylate cyclase by nitric oxide: regulation of enzyme activity by porphyrins and metalloporphyrins. Semin. Hematol. **26**: 63-76.

Irvine, R.F. (1990). 'Quantal' Ca^{2+} release and the control of Ca^{2+} entry by inositol phosphates--a possible mechanism. FEBS Lett. **263**: 5-9.

Jackson, S.P., S.M. Schoenwaelder, Y. Yuan, H.H. Salem and P. Cooray (1996). Non-receptor protein tyrosine kinases and phosphatases in human platelets. Thromb. Haemost. **76**: 640-650.

Jackson, T. (1991). Structure and function of G protein coupled receptors. Pharmacol. Ther. **50**: 425-442.

Jakobs, K.H., E. Bohme and S. Mocikat (1974). Cyclic GMP formation in human platelets. Naunyn-Schmeideberg Arch. Pharmacol. **282**: R40.

Jelsema, C. and J. Axelrod (1987). Stimulation of phospholipase A2 activity in bovine rod outer segments by the beta gamma subunits of tansducin and its inhibition by the alpha subunit. Proc. Natl. Acad. Sci. USA **84**: 3623-3627.

Johansson, J.S. and D.H. Haynes (1992). Cyclic GMP increases the rate of the calcium extrusion pump in intact human platelets but has no direct effect on the sense tubular calcium accumulation system. Biochim. Biophys. Acta **1105**: 40-50.

Kaibuchi, K., Y. Takai, M. Sawamura, M. Hoshijima, T. Fujikura and Y. Nishizuka (1983). Synergistic functions of protein phosphorylation and calcium mobilization in platelet activation. J. Biol. Chem. **258**: 6701-6704.

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.

Kasuya, J., H. Goko and Y. Fujita-Yamaguchi (1995). Multiple transcripts for the human cardiac form of the cGMP-inhibited cAMP phosphodiesterase. J. Biol. Chem. **270**: 14305-14312.

Kaupp, U.B. (1991) The cyclic nucleotide-gated channels of vertebrate photoreceptors and olfactory epithelium. Trends. Neurosci. **14**: 150-157.

Kawata, M., A. Kikuchi, M. Hoshijima, K. Yamamoto, E. Hashimoto, H. Yamamura and Y. Takai (1989). Phosphorylation of smg p21, a ras p21-like GTP-binding protein, by cyclic AMP-dependent protein kinase in a cell-free system and in response to prostaglandin E1 in intact human platelets. J. Biol. Chem. **264**: 15688-15695.

Kiefer, H.J., Lindstrom, J., Lennox, E.S. and S.J. Singer. (1970) Photo-affinity of specific acetylcholine-binding sites on membranes. Proc. Natl. Acad. Sci. USA **67**: 1688-1694.

Knighton, D.R., J. Zheng, R. Teneyck, V.A. Ashford, N.H. Xuong, S.S. Taylor and J.M. Sowadaski (1991). Crystal structure of the catalytic subunit of cyclic adenosine monophosphate-dependent protein kinase. Science **253**: 407-414.

Knowles, R. and S. Moncada (1994). Nitric oxide synthases in mammals. Biochem. J. **298**: 249-258.

Komalavilas, P. and T.M. Lincoln. (1996) Phosphorylation of the Inositol 1,4,5-trisphosphate receptor. J. Biol. Chem. **271**: 21933-21938.

Korschen, H., M. Illing, R. Seifer Sesti, F, A. Williams, S. Gotzes, C. Colville, F. Muller, A. Dose, M. Godde, L. Molday, et al. (1995). A 240 kDa protein represents the complete b-subunit of the cyclic nucleotide-gated channel from rod photoreceptor.

Neuron **15**: 627-636.

Kramer, R., E. Roberts, B. Strifler and E. Johnstone (1995). Thrombin induces activation of p38 MAP kinase in human platelets. J. Biol. Chem. **270**: 27395-27398.

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.

Lagnado, L. and D. Baylor (1992). Signal flow in visual transduction. Neuron **8**: 995-1002.

Landgraf, W., R. Huillin, C. Gobel and F. Hofmann (1986). Phosphorylation of cGMP-dependent protein kinase increases the affinity for cyclic AMP. Eur. J. Biochem. **154**: 113-117.

Law, D.A., L. Nannizzi-Alaimo and D.R. Phillips (1996). Outside-in integrin signal transduction. J. Biol. Chem. **271**: 10811-10815.

LeBon, T.R., J. Kasuya, R.J. Paxton, P. Belfrage, S. Hockman, V.C. Manganiello, Fujita and Y. Y (1992). Purification and characterization of guanosine 3′,5′-monophosphate-inhibited low K_m adenosine 3′,5′-monophosphate phosphodiesterase from human placental cytosolic fractions. Endocrinology **130**: 3265-3274.

Leigh, P.J., W.A. Cramp and J. MacDermot (1984). Identification of the prostacyclin receptor by radiation inactivation. J. Biol. Chem. **259**: 12431-12436.

Leroy, M.-J., E. Degerman, M. Taira, T. Murata, L.-H. Wang, M. Movsesian, E. Meacci and V. Manganiello (1996). Characterization of two recombinant PDE3 (cGMP-inhibited cyclic nucleotide phosphodiesterase) isoforms, RcGIP1 and HcGIP2, expressed NIH 3006 murine fibroblasts and Sf9 insect cells. Biochemistry **35**: 10194-10202.

Leung, L. and R. Nachman (1986). Molecular mechanisms of platelet aggregation. Annu. Rev. Med. **37**: 179-186.

Levin, R.I., B.B. Weksler and E.A. Jaffee (1982). The interaction of sodium nitroprusside with human endothelian cells and platelets: nitroprusside and prostacyclin synergistically inhibit platelet function. Circulation **66**: 1299-1307.

Levitzki, A. (1987). Regulation of adenylate cyclase by hormones and G-proteins. FEBS Lett. **211**: 113-8.

Lin, Y.M. and W.Y. Cheung (1980). in Calcium and cell function. W.Y. Cheung (eds). New York, Academic Press. Vol. 1. p. 79-100.

Lincoln, T.M., C.L. Hall, C.R. Park and J.D. Corbin (1976). Guanosine 3':5'-cyclic monophosphate binding proteins in rat tissues. Proc. Natl. Acad. Sci. USA **73**: 2559-2563.

Lindgren, S., A. Rascon, E. Degerman, P. Belfrage and V. Manganiello (1990). Identification of the cGMP-inhibited low K_m cAMP phosphodiesterase in rat aortic smooth muscle. Eur. J. Pharmacol. **183**: 806.

Lopez-Aparicio, P., A. Rascon, V.C. Manganiello, K.E. Andersson, P. Belfrage and E. Degerman (1992). Insulin induced phosphorylation and activation of the cGMP-inhibited cAMP phosphodiesterase in human platelets. Biochem. Biophys. Res. Commun. **186**: 517-523.

Loten, E.G., F.D. Assimacopoulos-Jeannet, J.H. Exton and C.R. Park (1978). Stimulation of a low K_m phosphodiesterase from liver by insulin and glucagon. J. Biol. Chem. **253**: 746-757.

Loten, E.G. and J.G. Sneyd (1970). An effect of insulin on adipose-tissue adenosine 3':5'-cyclic monophosphate phosphodiesterase. Biochem. J. **120**: 187-193.

Loughney, K., T. Martins, B. Sonnenburg and J. Beavo (1994). Isolation and characterization of cDNAs corresponding to two human clamodulin stimulated 3',5' cyclic nucleotide phosphodiesterase genes (Abstract). FASEB J. **8**: A469.

Lugnier, C. and N. Komas (1993). Modulation of vascular cyclic nucleotide phosphodiesterases by cyclic GMP: role in vasodilatation. Eur. Heart J. **14**(suppl.I): 141-148.

MacFarland, R., B. 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.

MacPhee, C., D. Reifsnyder, T. Moore, K. Lerea and J.A. Beavo (1988). Phosphorylation results in activation of a cAMP phosphodiesterase in human platelets. J. Biol. Chem. **263**: 10353-10358.

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. USA **83**: 6660-6663.

Manganiello, V., T. Tanaka and S. Murashima (1990b). Cyclic GMP-stimulated cyclic nucleotide phosphodiesterases. in Cyclic nucleotide phosphodiesterases:structure,

regulation and drug action. J. Beavo and M. Houslay (eds). New York, Wiley & Sons. Vol. 2. p. 61-86.

Manganiello, V.C., C.J. Smith, E. Degerman and P. Belfrage (1990a). Cyclic GMP-inhibited Cyclic Nucleotide Phosphodiesterases. in Cyclic Nucleotide Phosphodiesterases: Structure, regulation and drug action. J. Beavo and M. Houslay (eds). New York, John Wiley & Sons. Vol. 2. p. 87-117.

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.

Margulis, A., R.M. Goraczniak, T. Duda, R.K. Sharma and A. Sitaramayya (1993). Structural and biochemical identity of retinal rod outer segment membrane guanylate cyclase. Biochem. Biophys. Res. Commun. **194**: 855-861.

Markert, T., A.B. Vaandrager, S. Gambaryan, D. Pohler, C. Hausler, U. Walter, H.R. De Jonge, T. Jarchau and S.M. Lohmann (1995). Endogenous expression of type II cGMP-dependent protein kinase mRNA and protein in rat intestine. Implications for cystic fibrosis transmembrane conductance regulator. J. Clin. Invest. **96**: 822-830.

Marquis, N.R., J.A. Becker and R.L. Vigdahl (1970). Platelet aggregation. III. An epinephrine induced decrease in cyclic AMP synthesis. Biochem. Biophys. Res. Comm. **39**: 783-789.

Marquis, N.R., R.L. Vigdahl and P.A. Tavormina (1969). Platelet aggregation. I. Regulation by cyclic AMP and prostaglandin E1. Biochem. Biophys. Res. Comm. **36**: 965-972.

Martins, T.J., M.C. Mumby and J.A. Beavo (1982). Purification and characterization of a cyclic GMP-stimulated cyclic nucleotide phosphodiesterase from bovine tissues. J. Biol. Chem. **257**: 1973-1979.

Masuoka, H., Ito, M., Sugioka, M., Kozeki, H., Konishi, T., Tanaka, T. and T. Nakano. (1993) Two isoforms of cGMP-inhibited cyclic nucleotide phosphodiesterases in human tissues distinguished by their responses to vesnarinone, a new cardiotonic agent. Biochem. Biophys. Res. Commun. **190**: 412-417.

Maurice, D.H., D. Crankshaw and R.J. Haslam (1991). Synergistic actions of nitrovasodilators and isoprenaline on rat aortic smooth muscle. Eur. J. Pharmacol. **192**: 235-242.

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.

McAllister-Lucas, L.M., W.K. Sonnenburg, A. Kadlecek, D. Seger, H.L. Trong, J.L. Colbran, M.K. Thomas, K.A. Walsh, S.H. Francis, J.D. Corbin, et al. (1993). The structure of a bovine lung cGMP-binding, cGMP-specific phosphodiesterase deduced from a cDNA clone. J. Biol. Chem. **268**: 22863-22873.

McKean, M.L., J.B. Smith and M.J. Silver (1981). Formation of lysophosphatidylcholine by human platelets in response to thrombin. Support for the phospholipase A2 pathway for the liberation of arachidonic acid. J. Biol. Chem. **256**: 1522-1524.

Meacci, E., M. Taira, M. Moos Jr., C.J. Smith, M.A. Movsesian, E. Degerman, P. Belfrage and V. Manganiello (1992). Molecular cloning and expression of human myocardial cGMP-inhibited cAMP phosphodiesterase. Proc. Natl. Acad. Sci. USA **89**: 3721-3725.

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 guanine 3′,5′-monophosphate in ADP-induced huaman platelet aggregation in the presence of nitric oxide and related vasodilators. Blood **57**: 946-955.

Mellion, B.T., L.J. Ignarro, C.B. Myers, E.H. Ohlstein, B.A. Ballot, A.L. Hyman and P.J. Kadowitz (1983). Inhibition of human platelet aggregation by S-nitrosothiols: Heme-dependent activation of soluble guanylate cyclase and stimulation of cyclic GMP accumulation. Mol. Pharmacol. **23**: 653-664.

Mery, P.-F., C. Pavoine, F. Pecker and R. Fischmeister (1995). Erythro-9-(2-hydroxy-3-nonyl)adenine inhibits cyclic GMP-stimulated phosphodiesterase in isolated cardiac myocytes. Mol. Pharmacol. **48**: 121-130.

Michaeli, T., T.J. Bloom, T. Martins, K. Loughney, K. Ferguson, M. Riggs, L. Rodgers, J.A. Beavo and M. Wigler (1993). Isolation and characterization of a previously undetected human cAMP phosphodiesterase by complementation of cAMP phosphodiesterase-deficient Saccharomyces cerevisiae. J. Biol. Chem. **268**: 12925-12932.

Miot, F., P. Van Haastert and C. Erneux (1985). Specificity of cGMP binding to a purified cGMP-stimulated phosphodiesterase from adrenal tissue. Eur. J. Biochem. **149**: 59-65.

Miura, Y., K. Kaibuchi, T. Itoh, J.D. Corbin, S.H. Francis and Y. Takai (1992). Phosphorylation of smg p21B/rap1B p21 by cyclic GMP-dependent protein kinase. FEBS Lett **297**: 171-174.

Monaco, L., E. Vicini and M. Conti (1994). Structure of two rat genes coding for closely related rolipram-sensitive cAMP phosphodiesterases. Multiple mRNA variants originate from alternative splicing and multiple start sites. J. Biol. Chem. **269**: 347-357.

Moncada, S., R.M.J. Palmer and E.A. Higgs (1990). Relation between prostaglandin and nitric oxide in the thrombotic process. Thromb. Res. **Suppl. XI**:3-13.
Moro, M., R. Russell, S. Cellek, I. Lizasoain, Y. Su, V. Darley-Usmar, M. Radomski and S. Moncada (1996). cGMP mediates the vascular and platelet actions of nitric oxide: confirmation using an inhibitor of the soluble guanylyl cyclase. Proc. Natl. Acad. Sci. USA **93**: 1480-1485.

Muller, T., P. Engels and J. Fozard (1996). Subtypes of the type 4 cAMP phosphodiesterases: structure, regulation and selective inhibition. Trends in Pharm. Sci. **17**: 294-298.

Murashima, S., T. Tanaka, S. Hockman and V. Manganiello (1990). Characterization of particulate cyclic nucleotide phosphodiesterases from bovine brain: purification of a distinct cGMP-stimulated isoenzyme. Biochemistry **29**: 5285-5292.

Nakamura, K., M. Kimura and A. Aviv (1995). Role of cyclic nucleotides in store-mediated external Ca^{2+} entry in human platelets. Biochem. J. **310**: 263-269.

Nishikawa, M., M. Kanamori and H. Hioaka (1982). Inhibition of platelet aggregation and stimulation of guanylate cyclase by an antianginal agent molsidomine and its metabolites. J. Pharmacol. Exp. Ther. **220**: 183-190.

Nishizuka, Y. (1984). The role of protein kinase C in cell surface signal transduction and tumour promotion. Nature **308**: 693-698.

Novack, J.P., H. Charbonneau, J.K. Bentley, K.A. Walsh and J.A. Beavo (1991). Sequence comparison of the 63-, 61-, and 59-kDa calmodulin-dependent cyclic nucleotide phosphodiesterases. Biochemistry **30**: 7940-7947.

Pillai, R., Staub, S. and J. Colicelli. (1994) Mutational mapping of kinetic and pharmacological properties of a human cardiac cAMP phosphodiesterase. J. Biol. Chem. **269**: 30676-30681.

Polanowska-Grabowska, R. and A.R.L. Gear (1994). Role of cyclic nucleotides in rapid platelet adhesion to collagen. Blood **83**: 2508-2515.

Pyne, N. (1994). ASPET Multiple PDE Colloquium, New Port Beach, CA.

Pyne, N.J., M.E. Cooper and M.D. Houslay (1987). The insulin- and glucagon-stimulated 'dense-vesicle' high-affinity cyclic AMP phosphodiesterase from

rat liver. Purification, characterization and inhibitor sensitivity. Biochem. J. **242**: 33-42.

Radomski, M., R. Palmer and S. Moncada (1987). The role of nitric oxide and cGMP in platelet adhesion to vascular endothelium. Biochem. Biophys. Res. Comm. **148**: 1482-1489.

Radomski, M.W., R.M. Palmer and S. Moncada (1990). An L-arginine/nitric oxide pathway present in human platelets regulates aggregation. Proc. Natl. Acad. Sci. USA **87**: 5193-5197.

Radomski, M.W., R.M.J. Palmer and S. Moncada (1987). The anti-aggregating properties of vascular endothelium: interactions between prostacyclin and nitric oxide. Br. J. Pharmacol. **92**: 639-646.

Rahn, T., M. Ridderstrale, H. Tornqvist, V. Manganiello, G. Fredrikson, P. Belfrage and E. Degerman (1994). Essential role of phosphatidylinositol 3-kinase in insulin-induced activation and phosphorylation of the cGMP-inhibited cAMP phosphodiesterase in rat adipocytes. Studies using the selective inhibitor wortmannin. FEBS Lett. **350**: 314-318.

Rahn, T., L. Ronnstrand, M.-J. Leroy, C. Wernstedt, H. Tornqvist, V. Manganiello, P. Belfrage and E. Degerman (1996). Identification of the site in the cGMP-inhibited phosphodiesterase phosphorylated in adipocytes in response to insulin and isoproterenol. J. Biol. Chem. **271**: 11575-11580.

Reinhard, M., K. Giehl, K. Abel, C. Haffner, T. Jarchau, V. Hoppe, B.M. Jockusch and U. Walter (1995). The proline-rich focal adhesion and microfilament protein VASP is a ligand for profilins. EMBO J. **14**: 1583-1589.

Reinhard, M., M. Halbrugge, U. Scheer, C. Wiegand, B.M. Jockusch and U. Walter (1992). The 46/50 kDa phosphoprotein VASP purified from human platelets is a novel protein associated with actin filaments and focal contacts. EMBO J. **11**: 2063-2070.

Repaske, D.R., J.V. Swinnen, S.L. Jin, J.J. Van Wyk and M. Conti (1992). A polymerase chain reaction strategy to identify and clone cyclic nucleotide phosphodiesterase cDNAs. Molecular cloning of the cDNA encoding the 63-kDa calmodulin-dependent phosphodiesterase. J. Biol. Chem. **267**: 18683-18688.

Robichon, A. (1991). A new cGMP phosphodiesterase isolated from bovine platelets is substrate for cAMP- and cGMP-dependent protein kinases: evidence for a key role in the process of platelet activation. J. Cell. Biochem. **47**: 147-57.

Rowley, R.B., A.L. Burkhardt, H.-G. Chao, G.R. Matsueda and J.B. Bolen (1995). Syk protein-typrosine kinase is regulated by tyrosine-phosphorylated Igα/Igβ

immunoreceptor tyrosine activation motif binding and autophosphorylation. J. Biol. Chem. **270**: 11590-11595.

Ruggeri, Z.M. (1993). Mechanisms of shear-induced platelet adhesion and aggregation. Thromb. Haemost. **70**: 119-123.

Sage, S.O., R. Rease 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 intracellur Ca^{2+} store. Biochem. J. **265**: 675-680.

Salama, S.E. and R.J. Haslam (1981). Subcellular distribution of cyclic AMP-dependent protein kinase activity and of cyclic AMP-binding proteins in human platelets. FEBS Lett. **130**: 230-234.

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.

Salzman, E.W. (1972). Cyclic AMP and platelet function. New Engl. J. Med. **286**: 358-363.

Salzman, E.W. and L. Levine (1971). Cyclic 3',5'-adenosine monophosphate in human blood platelets. II. Effect of N^6-2'-o-dibutyryl cyclic 3',5'-adenosine monophosphate on platelet function. J. Clin. Invest. **50**: 131-141.

Sargeant, P., W.D. Clarkson, S.O. Sage and J.W.M. Heemskerk (1992). Calcium influx evoked by Ca^{2+} store depletion in human platelets is more susceptible to cytochrome P-450 inhibitors than receptor-mediated calcium entry. Cell Calcium **13**: 553-564.

Scott, J. (1991). Cyclic nucleotide-dependent protein kinases. Pharmac. Ther. **50**: 123-145.

Sette, C. and M. Conti (1996). Phosphorylation and activation of a cAMP-specific phosphodiesterase by the cAMP-dependent protein kinase. J. Biol. Chem. **271**: 16526-16534.

Sette, C., S. Iona and M. Conti (1994a). The short-term activation of a rolipram-sensitive, cAMP-specific phosphodiesterase by thyroid-stimulating hormone in thyroid FRTL-5 cells is mediated by a cAMP-dependent phosphorylation. J. Biol. Chem. **269**: 9245-9252.

Sette, C., E. Vicini and M. Conti (1994b). The ratPDE3/IVd phosphodiesterase gene codes for multiple proteins differentially activated by cAMP-dependent protein kinase. J. Biol. Chem. **269**: 18271-18274.

Shabb, J., L. Ng and J. Corbin (1990). One amino acid change produces a high affinity cGMP-binding site in cAMP-dependent protein kianse. J. Biol. Chem. **265**: 16031-16034.

Sharma, R.K. (1991). Phosphorylation and characterization of bovine heart calmodulin-dependent phosphodiesterase. Biochemistry **30**: 5963-5968.

Sharma, R.K., A.M. Adachi, K. Adachi and J.H. Wang (1984). Demonstration of bovine brain calmodulin-dependent cyclic nucleotide phosphodiesterase isozymes by monoclonal antibodies. J. Biol. Chem. **259**: 9248-9254.

Sharma, R.K. and J.H. Wang (1985). Differential regulation of bovine brain calmodulin-dependent cyclic nucleotide phosphodiesterase isoenzymes by cyclic AMP-dependent protein kinase and calmodulin-dependent phosphatase. Proc. Natl. Acad. Sci. USA **82**: 2603-2607.

Sharma, R.K. and J.H. Wang (1986). Regulation of cAMP concentration by calmodulin-dependent cyclic nucleotide phosphodiesterase. Biochem. Cell. Biol. **64**: 1072-1080.

Shattil, S.J. and L.F. Brass (1987). Induction of the fibrinogen receptor on human platelets by intracellular mediators. J. Biol. Chem. **262**: 992-1000.

Shibata, H., F.W. Robinson, T.R. Soderling and T. Kono (1991). Effects of okadaic acid on insulin-sensitive cAMP phosphodiesterase in rat adipocytes. Evidence that insulin may stimulate the enzyme by phosphorylation. J. Biol. Chem. **266**: 17948-17953.

Siess, W. (1989). Molecular mechanisms of platelet activation. Physiol. Rev. **69**: 58-178.

Siess, W. (1991). Multiple signal-transduction pathways synergize in platelet activation. NIPS **6**: 51-56.

Siess, W. and B. Grunberg (1993). Phosphorylation of rap 1b by protein kinase A is not involved in platelet inhibition of by cyclic AMP. Cell. Signal. **5**: 209-214.

Siess, W., D.A. Winegar and E.G. Lapetina (1990). Rap1-B is phosphorylated by protein kinase A in intact human platelets. Biochem. Biophys. Res. Commun. **170**: 944-950.

Simpson, A., M. Reeves and T. Rink (1988). Effects of SK&F 94120, an inhibitor of cyclic nucleotide phosphodiesterase type III, on human platelets. Biochem. Pharmacol. **37**: 2315-2320.

Smith, C.J., Francis, S.H., Walsh, K.A., Kurmar, S. and J.D. Corbin. (1996) Autophosphorylation of type 1β cGMP-dependent protein kinase increases basal catalytic activity and enhances allosteric activation by cGMP or cAMP. J. Biol. Chem. **271**: 20756-20762.

Smith, C.J., J. Krall, V.C. Manganiello and M.A. Movsesian (1993). Cytosolic and sarcoplasmic reticulum-associated low K_m, cGMP-inhibited cAMP phosphodiesterase in mammalian myocardium. Biochem. Biophys. Res. Commun. **190**: 516-521.

Smith, C.J., V. Vasta, E. Degerman, P. Belfrage and V.C. Manganiello (1991). Hormone-sensitive cyclic GMP-inhibited cyclic AMP phosphodiesterase in rat adipocytes. Regulation of insulin- and cAMP-dependent activation by phosphorylation. J. Biol. Chem. **266**: 13385-13390.

Sonnenburg, W.K., P.J. Mullaney and J.A. Beavo (1991). Molecular cloning of a cyclic GMP-stimulated cyclic nucleotide phosphodiesterase cDNA. Identification and distribution of isozyme variants. J. Biol. Chem. **266**: 17655-17661.

Sonnenburg, W.K., D. Seger and J.A. Beavo (1993). Molecular cloning of a cDNA encoding the "61-kDa" calmodulin-stimulated cyclic nucleotide phosphodiesterase. Tissue-specific expression of structurally related isoforms. J. Biol. Chem. **268**: 645-652.

Spiegel, A.M. (1987). Signal transduction by guanine nucleotide binding proteins. Mol. Cell. Endocrinol. **49**: 1-16.

Stroop, S.D., H. Charbonneau and J.A. Beavo (1989). Direct photolabeling of the cGMP-stimulated cyclic nucleotide phosphodiesterase. J. Biol. Chem. **264**: 13718-13725.

Stryer, L. (1991). Visual excitation and recovery. J. Biol. Chem. **266**: 10711-10714.

Sunahara, R., C. Dessauer and A. Gilman (1996). Complexity and diversity of mammalian adenylyl cyclases. Annu. Rev. Pharmacol. Toxicol. **36**: 461-480.

Surks, H., B. Sun, Y. Zhu, W. Baur, J. Hartwig and M. Mendelsohn (1995). Identification and cloning of a cGMP-gated ion channel from human platelets and megakaryocytes. (Abstract) Circulation **92**: I-36: 0168.

Suttorp, N., S. Hippenstiel, M. Fuhrmann, M. Krull and T. Podzuweit (1996). Role of nitric oxide and phosphodiesterase isoenzyme II for reduction of endothelial hyperpermeability. Am. J. Physiol. **270**: C778-C785.

Taira, M., S.C. Hockman, J.C. Calvo, M. Taira, P. Belfrage and V.C. Manganiello (1993). Molecular cloning of the rat adipocyte hormone-sensitive cyclic GMP-inhibited

cyclic nucleotide phosphodiesterase. J. Biol. Chem. **268**: 18573-18579.

Takemoto, D.J., K. Gonzalez, I. Udovichenko and J. Cunnick (1993). Cyclic GMP-regulated cyclic nucleotide phosphodiesterases. Cell. Signal. **5**: 549-553.

Tang, W.J., J. Krupinski and A.G. Gilman (1991). Expression and characterization of calmodulin-activated (type I) adenylylcyclase. J. Biol. Chem. **266**: 8595-8603.

Thomas, M.K., S.H. Francis and J.D. Corbin (1990). Characterization of a purified bovine lung cGMP-binding cGMP phosphodiesterase. J. Biol. Chem. **265**: 14964-14970.

Thompson, W.J. and M.M. Appleman (1971). Multiple cyclic nucleotide phosphodiesterase activities from rat brain. Biochemistry **10**: 311-316.

Tohmatsu, T., A. Nishida, S. Nagao, S. Nakashima and Y. Nozawa (1989). Inhibitory action of cyclic AMP on inositol 1,4,5-trisphosphate-induced Ca^{2+} release in saponin-permeabilized platelets. Biochim. Biophys. Acta. **1013**: 190-193.

Trong, H.L., N. Beier, W.K. Sonnenburg, S.D. Stroop, K.A. Walsh, J.A. Beavo and H. Charbonneau (1990). Amino acid sequence of the cyclic GMP stimulated cyclic nucleotide phosphodiesterase from bovine heart. Biochemistry **29**: 10280-12088.

Tsuboi, S., H. Matsumoto and A. Yamazaki (1994). Phosphorylation of an inhibitory subunit of cGMP phosphodiesterase in Rana catesbeiana rod photoreceptors. II. A possible mechanism for the turnoff of cGMP phosphodiesterase without GTP hydrolysis. J. Biol. Chem. **269**: 15016-15023.

Udovichenko, I.P., J. Cunnick, K. Gonzalez and D.J. Takemoto (1994). Functional effect of phosphorylation of the photoreceptor phosphodiesterase inhibitory subunit by protein kinase C. J. Biol. Chem. **269**: 9850-9856.

Vaandrager, A. and H. De Jonge (1996). Signalling by cAMP-dependent protein kinases. Mol. Cell. Biochem. **157**: 23-30.

Vaandrager, A.B. and H.R. De Jonge (1994). Effect of cyclic GMP on intestinal transport. Adv. Pharmacol. **26**: 253-83.

Venuti, M.C., R.A. Stephenson, R. Alvarez, J.J. Bruno and A.M. Strosberg (1988). Inhibitors of cyclic AMP phosphodiesterase. 3. Synthesis and biological evaluation of Pyrido and Imazolyl Analogues of 1,2,3,5-tetrahydro-2- oxoimidazo[2,1-b]quinazoline. J. Med. Chem. **31**: 2136-2145.

Verma, A., D.J. Hirsch, C.E. Glatt, G.V. Ronnett and S.H. Snyder (1993). Carbon monoxide: a putative neural messenger. Science **259**: 381-384.

Vorherr, T., L. Knopfel, F. Hofmann, S. Mollner, T. Pfeuffer and E. Carafoli (1993). The calmodulin binding domain of nitric oxide synthase and adenylyl cyclase. Biochemistry **32**: 6081-6088.

Vu, T.K., D.T. Hung, V.I. Wheaton and S.R. Coughlin (1991). Molecular cloning of a functional thrombin receptor reveals a novel proteolytic mechanism of receptor activation. Cell **64**: 1057-1068.

Walker, T.R. and S.P. Watson (1993). Synergy between Ca^{2+} and protein kinase C is the major factor in determining the level of secretion from human platelets. Biochem. J. **289**: 277-282.

Walter, U. (1989). Physiological role of cGMP and cGMP-dependent protein kinase in the cardiovascular system. Rev. Physiol. Biochem. Pharmacol. **113**: 41-88.

Wang, F., U.P. Naik, Y.H. Ehrlich, S. Osada, S. Ohno and E. Kornecki (1995). Stimulatory antibody-induced activation and selective translocation of protein kinase C isozymes in human platelets. Biochem. J. **311**: 401-406.

Wang, J.H., R.K. Sharma and M.J. Mooibroek (1990). Calmodulin-stimulated Cyclic nucleotide phosphodiesterases. in Cyclic nucleotide phosphodiesterases:structure, regulation and drug action. J. Beavo and M.D. Houslay (eds). New York, John Wiley & Sons. Vol. 2. p. 19-60.

Wang, X., C. Han and R. Fiscus (1991). Calcitonin gene-related peptide (CGRP) causes endothelium-dependent cyclic AMP, cyclic GMP and vasorelaxant responses in rat abdominal aorta. Neuropeptides **20**: 115-124.

Watson, N., M. Linder, K. Druey, J. Kehrl and K. Blumer (1996). RGS family members: GTPase-activating proteins for heterotrimeric G-protein alpha-subunits. Nature **383**: 172-175.

Wedel, B., P. Humbert, C. Harteneck, J. Foerster, J. Malkewitz, E. Bohme, G. Schultz and D. Koesling (1994). Mutation of His-105 in the beta 1 subunit yields a nitric oxide-insensitive form of soluble guanylyl cyclase. Proc. Natl. Acad. Sci. USA **91**: 2592-2596.

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.

Weiss, A. and D.R. Littman (1994). Signal transduction by lymphocyte antigen receptors. Cell **76**: 263-274.

Whalin, M.E., S.J. Strada and W.J. Thompson (1988). Purification and partial characterization of membrane-associated type II (cGMP-activatable) cyclic nucleotide phosphodiesterase from rabbit brain. Biochim. Biophys. Acta. **972**: 79-94.

White, J.G. (1987). An overview of platelet structural physiology. Scan. Microscopy **1**: 1677-1700.

Wolfe, S.M. and N. R. Shulman (1969). Adenyl cyclase activity in human platelets. Biochem. Biophys. Res. Comm. **35**: 265-272.

Wu, Z., R.K. Sharma and J.H. Wang (1992). Catalytic and regulatory properties of calmodulin-stimulated phosphodiesterase isozymes. Adv. Second Messenger Phosphoprotein Res. **25**: 29-43.

Yamamoto, T., V.C. Manganiello and M. Vaughan (1983). Purification and characterization of cyclic GMP-stimulated cyclic nucleotide phosphodiesterase from calf liver: effects of divalent cations on activity. J. Biol. Chem. **258**: 12526-12533.

Yamanishi, J., T. Yoshimi, K. Kaibushi, K. Sano, M. Castagna and Y. Nishizuka (1983). Synergistic functions of phorbol ester and calcium in serotonin release from human platelets. Biochem. Biophys. Res. Comm. **112**: 778-786.

Yamazaki, A., M. Yamazaki, V.A. Bondarenko and H. Matsumoto (1996). Discrimination of two functions of photoreceptor cGMP phosphodiesterase γ-subunit.

Yan, C. and J. Beavo (1994). Molecular cloning and cellular distribution of the 75 kDa calmodulin-dependent phosphodiesterase. (Abstract). FASEB J. **8**: A81.

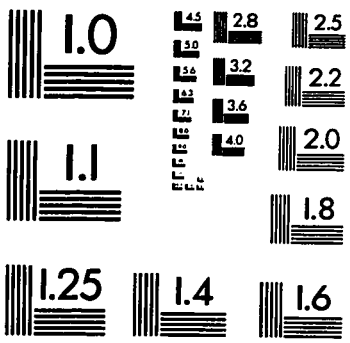
Yang, Q., M. Paskind, G. Bolger, W.J. Thompson, D.R. Repaske, L.S. Cutler and P.M. Epstein (1994). A novel cyclic GMP stimulated phosphodiesterase from rat brain. Biochem. Biophys. Res. Commun. **205**: 1850-1858.

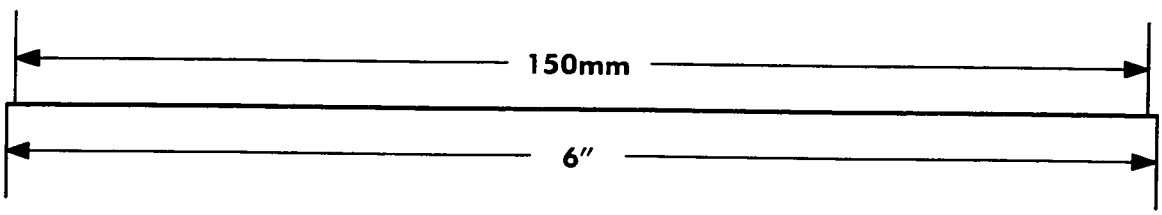
Yuen, P.S., L.R. Potter and D.L. Garbers (1990). A new form of guanylyl cyclase is preferentially expressed in rat kidney. Biochemistry **29**: 10872-10878.

Zimmerman, A. (1995). Cyclic nucleotide gated channels. Curr. Opin. Neurobiol. **5**: 296-303.

Zucker, M.B. and V.T. Nachmias (1985). Platelet activation. Arteriosclerosis **5**: 2-18.

Zufall, F., S. Firestein and G.M. Shepherd (1994). Cyclic nucleotide-gated ion channels and sensory transduction in olfactory receptor neurons. Annu. Rev. Biophys. Biomol. Struct. **23**: 577-607.





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