REGULATION OF HUMAN PLATELET CYCLIC NUCLEOTIDES

AND PLATELET AGGREGATION

BY cGMP-STIMULATED PHOSPHODIESTERASE

By

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ABSTRACT

Cyclic nucleotides are important inhibitory regulators of platelet function.

These second messengers are hydrolysed by cyclic $3',5'$-nucleotide phosphodiesterases

(PDEs). Three PDEs have been detected in human platelets: cGMP-stimulated

phosphodiesterase (PDE2), cGMP-inhibited phosphodiesterase (PDE3), and cGMP-

binding, cGMP-selective phosphodiesterase (PDE5). This research investigates the

contribution of PDE2 to the regulation of platelet cyclic nucleotide concentrations, and

the effects that PDE2 activity has on the inhibition by cAMP and cGMP of platelet

aggregation in response to thrombin or collagen. Increases in platelet cAMP were

initiated by stimulation of adenylyl cyclase with prostacyclin (PGI_2), whereas the

accumulation of cGMP was induced by nitroprusside (NP). The contributions of PDE2

to the hydrolysis of these cyclic nucleotides were evaluated using a novel inhibitor of the

enzyme, *erythro*-9-(2-hydroxy-3-nonyl)adenine (EHNA).

Before EHNA was used in experiments on platelet function, its effects on

partially purified preparations of the three platelet PDEs were studied. These

investigations demonstrated that EHNA is a selective and potent inhibitor of platelet

PDE2, and indicated that this compound is a more effective inhibitor of cAMP hydrolysis

in the presence than in the absence of cGMP.

To measure changes in cyclic nucleotide concentrations, platelets were

preincubated with [^{3}H]adenine and [^{3}H]guanine to label the metabolic nucleotide pools.

NP caused large concentration-dependent increases in platelet [^{3}H]cGMP levels, and this was associated with highly significant but much smaller increases in [^{3}H]cAMP accumulation, which were optimal with 10 μM NP. Higher concentrations of NP had much less effect on platelet [^{3}H]cAMP. A previous study had shown that the increases in platelet cAMP caused by NP were attributable to the inhibition of PDE3 by cGMP (Maurice and Haslam, 1990a), but the inhibitory component observed with high concentrations of NP had not been explained. The present research showed that the accumulation of cAMP and cGMP induced by high NP concentrations is enhanced by EHNA, and so provides the first demonstration that PDE2 activity restricts NP-induced cyclic nucleotide accumulation.

To assess whether these changes in platelet cyclic nucleotide levels were important, platelet aggregation in response to thrombin and collagen was monitored. In these studies, EHNA markedly increased the inhibitory action of NP on platelet aggregation. All the effects of NP on cyclic nucleotide accumulation and on platelet aggregation were blocked by a guanylyl cyclase inhibitor, 1H-[1,2,4]oxadiazolo [4,3-a]quinoxalin-1-one, confirming that NP acts solely through activation of this enzyme and that the increases in cAMP are secondary to cGMP formation. However, experiments with the adenylyl cyclase inhibitor, 2′,5′-dideoxyadenosine, which diminished the accumulation of cAMP but not that of cGMP, indicated that the inhibition of platelet aggregation is more closely correlated with the increases in cAMP than with

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those in cGMP. In experiments in which platelet PDE3 was selectively blocked by lixazinone, the accumulation of [^{3}H]cAMP was greatly increased and a corresponding inhibition of thrombin-induced platelet aggregation was observed. Both of these effects were greatly diminished when PDE2 was stimulated by NP (or cGMP). This research demonstrates for the first time that activation of PDE2 by cGMP has marked effects on platelet function, restricting the inhibition of platelet aggregation by agents that increase platelet cAMP.

To investigate the importance of PDE2 in regulating different platelet cAMP levels, the effects of EHNA were studied in the presence of 1 or 20 nM PGI$_2$. Whereas no significant increase in cAMP accumulation was caused by EHNA in the presence of 1 nM PGI$_2$, at the higher PGI$_2$ concentration a marked increase was detected when PDE2 was inhibited. NP potentiated the increase in cAMP seen with low PGI$_2$ but inhibited that seen with a high PGI$_2$ concentration, indicating a shift in the relative importance of PDE3 and PDE2 as platelet cAMP was increased. These studies show that in the presence of a high concentration of cAMP alone, or of regulatory cGMP, PDE2 makes a major contribution to the hydrolysis of platelet cAMP. Moreover, the results suggest that PDE2 inhibitors could be of value in the therapeutic modification of platelet responses.

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There are two instances in this thesis in which I present data from experiments that were in fact performed by Elliot K. Jang. This occurs on p. 92, in which I participated in the experiment presented in Figure 9 but the experiments which generated the supporting statistics (p. 93) were performed entirely by E. Jang. Also, the experiments relating to Table 3 (p. 117) were carried out by E. Jang.

Finally, I would like to acknowledge the contribution of Howard Rundle and my family to this thesis. Their encouragement and support have made all the difference.

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LIST OF ABBREVIATIONS

ACS   aqueous counting scintillant

ADA   adenosine deaminase

ADP   adenosine 5′-diphosphate

ANP   atrial natriuretic peptide

ATP   adenosine 5′-triphosphate

BSA   bovine serum albumin

$[Ca^{2+}]_i$ cytosolic free Ca^{2+} concentration

CaM   calmodulin

cAMP   adenosine cyclic 3′, 5′-monophosphate

cGMP   guanosine cyclic 3′, 5′-monophosphate

$cPLA_2$ cytosolic phospholipase A_2

DAG   *sn*-1,2-diacylglycerol

DDA   2′, 5′-dideoxyadenosine

EDTA   ethyleneglycol-bis-(β-aminoethyl ether) *N,N′*-tetraacetic acid

EHNA   *erythro*-9-(2-hydroxy-3-nonyl)adenine

G protein   guanine nucleotide binding protein

GAP   GTPase activating protein

GTP	guanosine 5′-triphosphate
Hepes	N-2-hydroxyethylpiperazine-N-2-ethanesulphonic acid
IBMX	3-isobutyl-1-methylxanthine
InsP$_3$	inositol 1,4,5-trisphosphate
MAPK	mitogen-activated protein kinase
MLCK	myosin light chain kinase
M$_r$	molecular weight
NP	nitroprusside
ODQ	1H-[1,2,4]oxadiazolo[4,3-a]quinoxalin-1-one
PDE	cyclic 3′,5′-nucleotide phosphodiesterase
PGI$_2$	prostacyclin
PIP$_2$	phosphatidylinositol 4, 5-bisphosphate
Pipes	piperazine-N,N′-bis(2-ethanesulphonic acid)
PKA	cAMP-dependent protein kinase
PKC	protein kinase C
PKG	cGMP-dependent protein kinase
PLA$_2$	phospholipase A$_2$
PLC	phospholipase C
PMSF	phenylmethylsulfonyl fluoride
PRP	platelet-rich plasma

PTX	pertussis toxin
SDS-PAGE	sodium dodecylsulphate polyacrylamide gel electrophoresis
TCA	trichloroacetic acid
TXA$_2$	thromboxane A$_2$
VASP	vasodilator-stimulated phosphoprotein
vWF	Von Willebrand factor

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1. INTRODUCTION

1.1. Platelet physiology

Platelets are small anuclear cells circulating in the blood. Their importance in the prevention of blood loss (haemostasis) cannot be overstated. Whereas one may survive defects in some blood clotting factors with relative ease, one could not last a single day without platelets (Jandl, 1996).

Platelets are formed from megakaryocytic precursors in the bone marrow. They receive all of their organelles (granules, mitochondria, membrane systems and ribosomes) from these progenitors (Jandl *et al.*, 1996, Kaushansky, 1995). Platelets lack nuclei and therefore do not synthesize mRNA (Shattil and Bennett, 1980). The shape and structure of platelets are maintained by two actin networks; an actin-myosin cytoskeleton supports internal structures and takes part in secretion of platelet granule constituents, and an actin-based membrane-associated skeleton supports platelet shape (reviewed by Zucker and Nachmias, 1985). Another structural element in platelets is the microtubule ring, composed of the protein tubulin, which also helps to maintain platelet shape (Zucker and Nachmias, 1985). There are two main membrane systems within platelets. The plasma membrane - composed of phospholipids, cholesterol and proteins - is invaginated to form the open canalicular system, a network of surface-connected channels which

provides an interface for the exchange of signals between platelets and their environment (Jandl *et al.*, 1996). Closely opposed to this system is found another membrane network, the dense tubular system, which stores the internal Ca^{2+} pool in a manner analogous to the smooth endoplasmic reticulum (Shattil and Bennett, 1980; Packham, 1994; Zucker and Nachmias, 1985). Within platelets are three types of secretory granules, dense granules contain ADP, ATP, Ca^{2+} and serotonin, α granules contain platelet-specific and plasma proteins and the lysosomal granules contain hydrolytic enzymes (Packham, 1994; Zucker and Nachmias, 1985).

The responses of platelets to stimulation follow a prescribed sequence and have been classified into primary and secondary responses. The former category refers to responses such as adhesion, shape change, and reversible aggregation; the latter category comprises granule secretion and irreversible aggregation (reviewed by Siess, 1989). The first response of platelets to stimulation is the development of adherence. The platelet-binding proteins, von Willebrand factor (vWF) and collagen, lie underneath the endothelial cell layer of blood vessels and are therefore normally inaccessible to platelets (Bennett, 1992). Damage to the endothelium exposes these proteins to platelets, which then adhere to vWF and are thereby brought into close proximity with collagen (Siess, 1989). Stimulation by collagen (or by other stimuli) then initiates a stereotyped sequence of responses from platelets (Jandl, 1996; Shattil and Bennett, 1980; Bennett, 1992). A change in their shape, from disc-like to spherical, ensues and platelets develop long, thin,

filopodial extensions from their main bodies (Jandl, 1996; Glenn *et al.*, 1996). In the presence of weak stimuli and of extracellular Ca^{2+}, platelets may form small and reversible aggregates, which involves the unmasking of the active form of the fibrinogen receptor and subsequent cross-linking of adjacent platelets by fibrinogen molecules (Siess, 1989). All of the events described thus far are potentially reversible reactions. The turning point between reversible and irreversible aggregation of platelets is secretion, also known as the release reaction, which is induced by strong stimuli such as thrombin and collagen (Siess, 1989). Secretion from platelet granules releases ADP, serotonin and fibrinogen, all of which enhance platelet aggregation. Thus, irreversible aggregation is a positive feedback process which proceeds in cycles of stimulation and release. The resulting platelet plug is strengthened by thrombin, which catalyses the conversion of fibrinogen to fibrin (Packham, 1994).

Regulation of the platelet response is critical to haemostasis. On the one hand, an insufficient platelet response is the cause of several serious bleeding disorders: e. g. Bernard-Soulier syndrome, Glanzmann's thrombasthenia, and thrombocytopenias (Bennett, 1992; Bick, 1994). On the other hand, excessive platelet reactivity is also linked to serious health consequences such as myocardial infarction, sudden coronary death, and cerebrovascular accidents (Meade, 1992; Blockmans *et al.*, 1995).

1.2.   Signal transduction in the activation of platelets

1.2.1.   Introduction

Platelet responses are triggered by a variety of stimuli: by primary agonists (thrombin, collagen) at the site of vessel wall damage, by secondary agonists (ADP, serotonin, TXA_2) released by activated platelets, and by hormones such as epinephrine (reviewed by Brass *et al.*, 1994a). Although the signals which stimulate platelets are diverse, the responses that are provoked converge on two main pathways: activation of phospholipase C (PLC) and of phospholipase A_2 (PLA_2) (Brass *et al*, 1994a). Firstly, stimulation of PLC results in formation of inositol 1,4,5-trisphosphate ($InsP_3$) and 1,2-diacylglycerol (DAG), which subsequently cause the release of internal Ca^{2+} stores and activation of protein kinase C (PKC), respectively (Brass *et al.*, 1993 and 1994a; Kroll and Schafer, 1989). Secondly, PLA_2 releases arachidonic acid from membrane phospholipids, which is converted by cyclooxygenase enzymes to the prostaglandins PGG_2 and PGH_2; the latter is metabolized to TXA_2 which provides a further potent stimulus leading to the activation of PLC (Brass *et al.*, 1993 and 1994a; Blockmans *et al.*, 1995). Increases in platelet cAMP levels oppose many of these responses (Blockmans *et al.*, 1995; Ashby *et al.*, 1990). The signal transduction of platelet responses is outlined below. Where possible, the relevance of thrombin and collagen stimuli in these processes will be highlighted.

1.2.2.　Thrombin and collagen receptors

The activity of thrombin is critical to haemostasis; it activates coagulation factors, stimulates the procoagulant activity of platelets, and is a strong activator of platelet aggregation and secretion (Packham, 1994; Brass *et al.*, 1994b; Coughlin, 1994). There are approximately 50 high affinity (K_d 0.3 nM) and 1, 700 moderate affinity (K_d 11 nM) thrombin binding sites on human platelets (Brass *et al.*, 1993).

A receptor for thrombin has been cloned, and this was the first member of the tethered ligand receptor family to be described (Coughlin, 1994). The topology of this molecule is similar to other seven transmembrane receptors, but its mechanism of activation is quite different. When thrombin binds, it cleaves its receptor at position 41/42, which exposes a SFLLRN sequence at the fresh amino terminus (Coughlin, 1994). Peptides with this sequence interact with the first transmembrane helix and second extracellular loop of the receptor, causing activation of the receptor (Brass and Molino, 1997). In this way, the amino terminus is a tethered ligand that binds to the receptor site (Coughlin, 1994).

Antibodies directed against the cloned thrombin receptor have detected between 1, 500 and 2, 000 thrombin receptors on the surface of human platelets (reviewed by Brass and Molino, 1997). This closely agrees with the number of moderate-affinity binding sites, which is reported as 1, 700 for human platelets (reviewed by Brass *et al.*, 1993).

It is likely that there is more than one thrombin receptor on platelets, at least in mice. When the gene for the tethered ligand thrombin receptor was disrupted in mice, their platelets retained responsiveness to thrombin (Connolly *et al.*, 1996). This suggests a redundancy in thrombin receptors on mouse platelets; however, there have been no similar claims for human platelets (Brass *et al.*, 1997b).

In most cells, the thrombin receptor is inactivated by phosphorylation on the cytoplasmic domain (by a G-protein coupled receptor kinase) and receptor molecules are eventually internalized (Brass and Molino, 1997). Sensitivity to thrombin is then slowly recovered as new receptor molecules are synthesized (Brass *et al.*, 1994b). In platelets, however, very little protein is synthesized and fresh receptor molecules do not reappear at the plasma membrane surface. Therefore, once a platelet has been stimulated by thrombin, it remains refractory to this stimulus.

The connective tissue protein collagen is a strong activator of platelets (Blockmans *et al*, 1995). Collagen monomers are comprised of three helical polypeptide chains, which have a repetitive amino acid sequence GXY (X and Y are often proline) (Creighton, 1993). The monomers line up side-by-side to form microfibrils, which is the form of collagen that is required for platelet aggregation (Siess, 1989)

The binding of several platelet glycoproteins (designated gp) to collagen has been demonstrated, though it remains to be proven that any of these proteins are required for collagen-induced platelet aggregation (Siess, 1989). The gpIa/IIa (integrin $\alpha_2\beta_1$)

complex binds to collagen, and patients lacking this protein have a prolonged bleeding time; however, this effect has since been discovered to be due to a defect in adhesion to collagen, rather than aggregation (reviewed by Sixma *et al.*, 1995 and 1997). Another collagen-binding protein which may be a functional receptor is gpVI (reviewed by Moroi and Jung, 1997). GpIV is another collagen-binding protein that also binds to thrombospondin and may therefore be involved in a non-specific signalling mechanism (Hourani and Cusack, 1991). The roles of these collagen-binding proteins in platelet signal transduction have not yet been elucidated. In the presence of collagen, phosphorylation of syk tyrosine kinase and of PLCγ2 has been reported, although the relation of these phenomena to collagen binding is not clear (Sixma *et al.*, 1997; Moroi and Jung, 1997).

1.2.3.   G proteins involved in thrombin signalling

Seven transmembrane receptors accomplish their intracellular signalling by coupling to effector enzymes through heterotrimeric guanine nucleotide binding proteins (G proteins) (Brass *et al.*, 1993). At present, nine members of the G_s, G_i, G_q, G_z, and G_{12} families of G proteins have been identified in human platelets (Brass *et al.*, 1997). G proteins are activated by the exchange of their bound GDP for GTP. This releases the α subunit from the $\beta\gamma$ complex, and both of these moieties may then perform signalling functions (reviewed by Birnbaumer, 1992). The participation of G proteins has been

demonstrated in the signalling pathways of many platelet agonists.

The effects of thrombin are due to the actions of multiple G proteins. As will be described shortly, thrombin exerts inhibitory effects on adenylyl cyclase and stimulatory effects on phosphoinositide hydrolysis. Inhibition of adenylyl cyclase by thrombin is blocked by pertussis toxin (PTX), showing that this is mediated by the action of G_i (Houslay *et al.*, 1986). This action of thrombin on adenylyl cyclase is blocked by activation of PKC, suggesting that this kinase may inhibit $G_{i\alpha}$ (Williams *et al.*, 1987). The stimulatory effects of thrombin on phosphoinositide metabolism are partially blocked by PTX, indicating that G_i also plays a role in this pathway, presumably due to activation of PLCβ2 by the βγ complex of G_i (Brass *et al.*, 1997). The PTX-insensitive component of GTPase activity observed in response to thrombin may be due to coupling of the receptor to G_q (Benka *et al.*, 1995). Thus, the engagement of multiple pathways during thrombin stimulation is accomplished through the coupling of the receptor to diverse G proteins.

1.2.4. Effector enzymes

In platelets, one of the effector enzymes which may be stimulated by G proteins is PLC. When stimulated by the thrombin pathway, PLC cleaves the membrane phospholipid phosphatidylinositol 4, 5-bisphosphate (PIP$_2$) at the C-3 position (Kroll and Schafer, 1989; Ashby *et al*, 1990). From this precursor, two second messengers are

generated; InsP$_3$ diffuses away from the plasma membrane, leaving behind DAG (Kroll and Schafer, 1989; Ashby *et al.*, 1990). There are three groups of PLC enzymes, based on their structures and regulatory mechanisms: PLCβ family members are stimulated by G proteins, PLCγ by tyrosine phosphorylation, and PLCδ by an as yet unknown mechanism (Lee and Rhee, 1995; Nishizuka, 1995). Members of all three families have been discovered in platelets (Tate and Rittenhouse, 1993). The activation of various PLCβ family members is carried out by either the α or βγ subunits of G proteins: Gα$_q$ activates PLCβ$_1$, PLCβ$_2$, and PLCβ$_3$, whereas βγ activates PLCβ$_2$ and PLCβ$_3$ (Lee, and Rhee, 1995; Nishizuka, 1995; Birnbaumer, 1992). Thrombin stimulation also leads to activation of a tyrosine kinase pathway, the net result of which is tyrosine phosphorylation of PLC-γ$_2$ (Tate and Rittenhouse, 1993). However, PLC-γ$_2$ appears to offer only a minor contribution to total platelet PLC activity (Tate and Rittenhouse, 1993).

Stimulation of platelets (by thrombin or collagen) leads to the engagement of a second major signalling pathway which ends with the activation of cPLA$_2$ (Blockmans *et al.*, 1995). The regulation of this enzyme in platelet activation is not completely understood. The activity of cPLA$_2$ is strongly increased by Ca^{2+}, and in recent years this effect has been shown to be potentiated by phosphorylation (Kramer *et al.*, 1993; Börsch-Haubold, 1995). It has been demonstrated that p38 mitogen-activated protein kinase (MAPK) phosphorylates cPLA$_2$ when platelets are stimulated by thrombin (Kramer *et al.*,

1993). However, when the activity of p38 MAPK was blocked, phosphorylation of

$cPLA_2$ was only partially reduced, and only a mild inhibition of activity was observed,

suggesting that an as yet unidentified other kinase may also be acting on $cPLA_2$ (Börsch-

Haubold, 1995; Kramer *et al.*, 1996). Activated PLA_2 releases arachidonic acid (AA)

from dense tubular membranes (Blockmans *et al.*, 1995). This molecule is metabolized

into prostaglandins (such as PGD_2, PGG_2 and PGH_2) and from PGH_2 into TXA_2, which

diffuses out of the cell and strongly activates nearby platelets (Kroll and Schafer, 1989;

Ashby *et al.*, 1990).

1.2.5.   Second messengers involved in platelet activation

Two second messengers are produced by the action of PLC: $InsP_3$ and DAG.

The major effect exerted by $InsP_3$ is the release of stored Ca^{2+} - a crucial signalling

molecule in platelets - from the dense tubular system (Blockmans *et al.*, 1995; Kroll and

Schafer, 1989; Ashby *et al.*, 1990). The emptying of internal Ca^{2+} stores is associated

with increased Ca^{2+} influx across the plasma membrane (Blockmans *et al.*, 1995; Kroll

and Schafer, 1989; Ashby *et al.*, 1990).

Calcium has multiple effects within the platelet. When bound to calmodulin

(CaM), it activates myosin light chain kinase (MLCK) and thus the contractile response

of platelet myosin, which produces the tensile force required for centralization of the

platelet granules (Blockmans *et al.*, 1995; Kroll and Schafer, 1989; Ashby *et al.*, 1990).

Ca^{2+} also activates cytosolic PLA_2 ($cPLA_2$) and the Ca^{2+}-dependent proteases

(Blockmans *et al.*, 1995; Kroll and Schafer, 1989; Ashby *et al.*, 1990).

DAG - the other second messenger produced by PLC - activates PKC once it

has been targeted to the membrane by Ca^{2+} and acidic phospholipids (phosphatidyl serine

is preferred) (Newton, 1995). There are three classes of PKC isoforms: conventional

PKCs, which are regulated by Ca^{2+} and DAG; novel PKCs, which are regulated by DAG

but not Ca^{2+}; and atypical PKCs, which do not appear to respond to Ca^{2+}, DAG or

phorbol esters (Newton, 1995). Activated PKC phosphorylates multiple targets on serine

and threonine residues (Newton, 1995). The major substrate of platelet PKC is

pleckstrin, a 40 kDa protein which binds to $G\beta\gamma$ and to PIP_2 and appears to prevent

activation of PLC once phosphorylated (Abrams *et al.*, 1996; reviewed by Brass *et al.*,

1997). PKC can also phosphorylate the light chain of myosin at a site distinct from that

of MLCK, although it is not clear if this phosphorylation causes a physiological effect

(Zucker and Nachmias, 1985).

Another crucial second messenger in platelet signal transduction is cAMP.

Platelet responses are the result of the integration of a multiplicity of stimulatory and

inhibitory signals. The stimulatory signals have been outlined above. A major inhibitory

pathway in platelets is mediated by cAMP through its stimulation of cAMP-dependent

protein kinase (PKA) (Haslam *et al.* 1978; Ashby *et al.*, 1990). cAMP blocks the

multiple stimulatory effects of Ca^{2+} by lowering its cytosolic concentration (reviewed by

Haynes, 1993). Moreover, PKA opposes platelet activation by inhibiting MLCK and by indirect inhibitory effects on PKC activity (Kroll and Schafer, 1989) .

1.3. Platelet cyclic nucleotides

1.3.1. Adenine nucleotide pools in platelets

Because early research focused on the production of platelet ATP - required for responses such as shape change and secretion - far more is known about platelet adenine than guanine nucleotides (Holmsen, 1985). Platelets transport adenine and adenosine across the plasma membrane into the cytosol, where they are transformed into AMP by the actions of adenine phosphoribosyl transferase and adenosine kinase, respectively (Holmsen, 1985). AMP is then converted into ADP by the addition of inorganic phosphate by adenylate kinase (Holmsen, 1985).

Adenine nucleotides are localized within three compartments in platelets: the dense granule, cytoplasmic, and actin-bound pools (Holmsen, 1985). The actin-bound pool accounts for only 4% of platelet adenine nucleotides and consists entirely of ADP (Holmsen, 1985). The majority of adenine nucleotides (64%) are located within a slowly-exchanging pool in the dense granules (Holmsen, 1985). The cytosolic pool (32%) is the source of metabolic nucleotides, and is characterized by rapid turnover (Holmsen, 1985). Within this compartment, the concentrations of adenine nucleotides in resting platelets are: 6.7 mM ATP, 0.7 mM ADP, and 0.07 mM AMP (Holmsen, 1985; Eigenthaler *et al.*,

1992). Resting platelet cAMP, as determined by radioimmunoassay, amounts to only 18

pmol/10^9 platelets (Haslam *et al.*, 1980). This corresponds to a total cytosolic cAMP

concentration of approximately 3 μM; however, because the concentration of cAMP-

binding sites on the regulatory subunits of PKA is 6 μM in platelets, the cytosolic

concentration of free cAMP is approximately 0.1 μM (Eigenthaler *et al.*, 1992). A

doubling of the total cytosolic concentration of cAMP therefore leads to saturation of

binding sites on PKA, and a resulting activation of this kinase.

Due to the significant effects on platelet responses that even a slight increase in

platelet cAMP can cause, it is necessary to employ a highly sensitive method to measure

platelet cyclic nucleotides. In this laboratory, changes in cyclic nucleotides have been

measured using the prelabelling method, in which metabolic cyclic nucleotides are

labelled by incubation of platelets with [^{3}H]adenine and [^{3}H]guanine and subsequently

recovered using chromatographic techniques (Davies *et al.*, 1976; Maurice and Haslam,

1990a). The prelabelling method has been shown to give the same results as

radioimmunoassays but to be less subject to error (Maurice and Haslam, 1990a).

1.3.2.   Synthesis of cAMP

The formation of cAMP is governed by the adenylyl cyclases, an enzyme

family which contains at least ten members. All types of adenylyl cyclase are stimulated

by Gα_s and by forskolin. The regulation of individual types is complex. Examples of

stimulation by Gβγ, Ca-CaM, PKC and of inhibition by G_i, Ca^{2+}, $G_i\beta\gamma$ are observed in various combinations among the different types of adenylyl cyclase (reviewed by Taussig and Gilman, 1995).

Adenylyl cyclases contain twelve transmembrane segments and two large cytosolic domains, one intervening between the sixth and seventh transmembrane α-helix, and the other appearing at the carboxyl terminus of the enzyme (reviewed by Sunahara *et al.*, 1996). The amino terminal portions of each cytosolic domain (designated C1a and C2a, respectively) are highly conserved amongst family members, and furthermore resemble each other and the catalytic domains of soluble guanylyl cyclase (Taussig and Gilman, 1995). Expression of both C1 and C2 domains together, or of C2 alone, is sufficient for catalytic activity. Zhang *et al.* (1997) have recently solved the crystal structure of the rat type II adenylyl cyclase C2 region complexed with forskolin. The monomer is formed by a three layer αβ sandwich, which dimerizes through head-to-tail interactions. Formation of the dimer creates a prominent cleft, in which charged and polar residues mediate ATP binding. Because the primary sequences of C1 domains are similar to those of C2, the authors speculate that this structure may apply to the native molecule.

Several platelet agonists act by increasing platelet cAMP through a stimulatory coupling to adenylyl cyclase. An example is PGI_2, which is synthesized and secreted by endothelial cells (Ware and Heistad, 1993; Pearson, 1994). When activated platelets

release ADP, ATP or serotonin, the ensuing increase in endothelial cytosolic Ca^{2+} levels

activates endothelial PLA_2, which then releases arachidonic acid (Ware and Heistad,

1993; Pearson, 1994). The released AA is converted into PGI_2, which diffuses out of

endothelial cells and inhibits activation of nearby platelets through its stimulation of

adenylyl cyclase (Aiken *et al.*, 1979).

There are two types of PGI_2 binding sites in the platelet: relatively abundant

low-affinity sites (greater than 3, 000 binding sites, K_d 1 µM), and less common high-

affinity sites (between 100 and 3, 000 binding sites, K_d 10 nM) (Ashby *et al.*, 1990b;

Blockmans *et al.*, 1995). The receptor for PGI_2 (the IP receptor) has been cloned, and is a

member of the seven transmembrane G-protein-coupled receptor superfamily (Boie *et al.*,

1994). Binding of PGI_2 to the IP receptor leads to stimulation of adenylyl cyclase

through G_s (Ashby *et al.*, 1990b). Conversely, at high concentrations, binding of PGI_2 to

lower affinity receptor inhibits adenylyl cyclase through coupling of this unidentified

receptor to G_i (Ashby *et al.*, 1990b). In the present work, investigations into the relative

roles of platelet cAMP phosphodiesterases (PDEs) in the presence of different cAMP

concentrations have been performed, employing PGI_2 (1 or 20 nM) as an activator of

adenylyl cyclase.

Adenosine exerts a biphasic effect on platelet adenylyl cyclase, and non-

stimulatory analogues have been identified with an enhanced inhibitory action (Haslam

and Lynham, 1972; Haslam *et al.*, 1978b). These adenylyl cyclase inhibitors are now

referred to as "P site" inhibitors, because their purine rings must be intact for an inhibitory effect to be seen (Londos and Wolff, 1977). "P site" inhibitors are typically more effective against stimulated, rather than basal adenylyl cyclase activity (Haslam *et al.*, 1978b). One such compound, 2',5'-dideoxyadenosine (DDA), is a potent inhibitor of the PGE_1-stimulated form of the enzyme (IC_{50}, 4 μM) and has been used to block the inhibition of platelet aggregation by PGE_1 (Haslam *et al.*, 1978b). In the present study, DDA has been utilized to inhibit adenylyl cyclase in the platelet and therefore to prevent cAMP-dependent processes.

1.3.3.   Formation of cGMP

The second messenger cGMP is formed from GTP by guanylyl cyclase (reviewed by Drewett and Garbers, 1994). The main forms of these enzymes are membrane-bound and soluble guanylyl cyclases. The particulate forms of guanylyl cyclase are transmembrane receptors which bind ligands such as atrial natriuretic factor and speract. A type of guanylyl cyclase is expressed exclusively in the retina, where the cGMP that it synthesises opens cGMP-gated cation channels. In platelets, increases in cGMP are mediated solely by the soluble form of guanylyl cyclase, which is a heterodimer composed of α_1 (82 kDa) and β_1 (70 kDa) subunits (Buechler *et al.*, 1994). Both contain a carboxy terminal region which is homologous to the catalytic domains of particulate guanylyl cyclase and adenylyl cyclase (Taussig and Gilman, 1995). In the

platelet, soluble guanylyl cyclase is primarily stimulated by nitric oxide (NO), leading to inhibition of platelet aggregation (Buechler *et al.*, 1994; Moro *et al.*, 1996).

The signalling properties of NO have only been recognized in the last fifteen years (reviewed by Star, 1993). Endothelial cells produce and release NO when their $[Ca^{2+}]_i$ is increased (Griffith and Stuehr, 1995; Radomski *et al.*, 1987; Moncada *et al.*, 1991). NO diffuses out of endothelial cells and into nearby platelets, in which it activates soluble guanylyl cyclase. The ensuing increases in cGMP lead to inhibition of platelet activation (Moncada *et al.*, 1991).

Aside from its production by NO synthase, NO can also be chemically introduced into platelets by NO-donors, from which NO is released either spontaneously or enzymatically (Gerzer *et al.*, 1988). One such compound is nitroprusside, $[Fe(CN)_5NO]^{2-}$ (NP), which releases NO within platelet cytosol and stimulates guanylyl cyclase (Mellion *et al.*, 1983). It is thought that NO reacts with cytosolic thiol groups to form nitrosothiols, which then stimulate guanylyl cyclase through an interaction with the heme group associated with the enzyme (Gerzer *et al.*, 1988; Mellion *et al.*, 1983). A major part of the research presented in this work concerns the effects of cGMP on platelet cAMP levels and on platelet aggregation. In these studies, increases in cGMP were induced by incubating platelets with NP.

In 1995, ODQ was identified as a selective inhibitor of the soluble form of guanylyl cyclase (Garthwaite *et al.*, 1995). ODQ does not inhibit particulate guanylyl

cyclase, NO synthase, or adenylyl cyclase activity (Garthwaite *et al.*, 1995). Due to the lack of detailed studies on the kinetics of ODQ inhibition, only IC_{50} data is available; in the case of cerebellum slices, the cGMP response to glutamate receptor agonists was decreased 50% by 20 nM ODQ (Garthwaite *et al.*, 1995).

In the present study, NO-donors have been used to stimulate guanylyl cyclase activity. Because NO is a free radical and therefore a highly reactive molecule, it is conceivable that NO causes effects within the platelet other than stimulation of guanylyl cyclase. For example, NP stimulates the ADP-ribosylation of glyceraldehyde-3-phosphate dehydrogenase in erythrocytes and it is therefore possible that similar modifications of platelet proteins could be induced by NP (Kots *et al.*, 1992). Recently, a group has tested the effects of ODQ on platelet responses to NO and has concluded that with respect to collagen-induced platelet aggregation, the effects of NO are entirely attributable to its effects on cGMP (Moro *et al.*, 1996). In the present work, the effects of NP on collagen-induced platelet aggregation and cyclic nucleotide levels were investigated using ODQ.

1.3.4.   Effects on platelet function mediated by cyclic nucleotides

cAMP is an important negative regulator of platelet responses. Platelet inhibitors, such as PGE_1 and PGI_2, activate adenylyl cyclase and cause concentration-dependent increases in cAMP concentration (Haslam *et al.*, 1978; Brass *et al.*, 1991). At

the same time, many stimulatory agonists such as thrombin, ADP, and epinephrine diminish the increases in platelet cAMP levels caused by inhibitory agonists (Haslam *et al.*, 1978; Williams *et al.*, 1987).

Initially, the effects of cAMP on platelet function were poorly understood. It was well known that compounds such as PGE_1 inhibit platelet function by increasing cAMP (Wolfe and Shulman, 1969; Marquis *et al.*,1969; Ball *et al.*, 1970). When aggregating agents such as ADP were added simultaneously with PGE_1, a significant reduction in platelet cAMP accumulation was observed (Cole *et al.*, 1971). By extrapolation, these observations led to the hypothesis that a reduction in cAMP levels below resting levels might cause platelet aggregation. In this way, platelet cAMP was described as a bidirectional regulator of platelet function, meaning that changing the level of cAMP could either stimulate or inhibit platelet aggregation. This concept was subsequently refuted when it was clearly shown that inhibition of adenylyl cyclase in resting platelets does not in itself trigger platelet aggregation (Haslam *et al.*, 1978a, b). Thus, cAMP is a unidirectional regulator of platelet function; it can inhibit but not stimulate platelet aggregation.

Opinion concerning the role of cGMP in platelet function has changed considerably over the years. Initially, it was observed that increases in cGMP closely parallelled the extent of platelet aggregation observed in the presence of ADP, collagen, or serotonin, suggesting that cGMP may play a functional role in inducing platelet

aggregation (Davies *et al.*, 1976; Chiang *et al.*, 1976). Later analysis showed that the observed increases in cGMP were associated with early events in platelet aggregation, and were in fact an accompanying effect, rather than a cause, of platelet aggregation (Haslam *et al.*, 1978a). A stimulatory role for cGMP was further disproved by the observation that agents that increase cGMP, such as NP, inhibit platelet aggregation (Haslam *et al.*, 1978a, 1980; Mellion *et al.*, 1981).

1.3.5. Synergistic effects of stimulators of adenylyl and guanylyl cyclases

In 1982, it was observed that NP and PGI_2 have synergistic inhibitory effects on platelet aggregation. Whereas PGI_2 (3 nM) alone inhibited 32% of epinephrine-induced platelet aggregation, and NP (0.5 µg/ml) inhibited 17%, when these compounds were added simultaneously, 89% of platelet aggregation was prevented (Levin *et al.*, 1982).

This observation has since been reexamined using a variety of aggregating agents, such as adrenaline, collagen, and platelet activating factor. In many examples, NO, NP, or organic nitrates dose-dependently inhibited platelet aggregation and the inhibitory effects of PGI_2 were greatly increased by the addition of NO or NP; (Macdonald *et al.*, 1988; De Caterina *et al.*, 1988; Willis *et al.*, 1989). However, the mechanism underlying these effects was not clear.

In 1990, Maurice and Haslam showed that the synergistic inhibition of

aggregation observed in the presence of prostaglandins and NO donors was due to their combined effects on platelet cyclic nucleotides. In platelets, there are three cyclic $3',5'$-nucleotide phosphodiesterase (PDE) isoenzymes, one of which is PDE3, a cGMP-inhibited phosphodiesterase activity that primarily hydrolyses cAMP. Maurice and Haslam (1990) showed that increases in cGMP (caused by stimulation of guanylyl cyclase) inhibited the breakdown of cAMP by PDE3 in intact rabbit platelets. This effect enhanced the accumulation of cAMP induced by PGE_1, which was markedly increased in a concentration-dependent fashion by NP. In agreement with these observations, the inhibition of aggregation by PGE_1 was potentiated by NP. No such interaction between NP and PGE_1 was observed with respect to cGMP levels. The synergistic inhibition of aggregation by NP with PGE_1 was reduced by the adenylyl cyclase inhibitor, DDA, which also decreased the accumulation of cAMP (but not of cGMP). This suggested that the inhibition was caused by the effects of NP on cAMP rather than on cGMP accumulation. The mechanism of action of NP was further clarified by studying the action of cilostamide, an inhibitor of PDE3. Although NP strongly increased the cAMP accumulation observed in the presence of PGE_1, it did not increase that observed in the presence of cilostamide. This implicated PDE3 as the target of the action of NP (or cGMP).

The effects described by Maurice and Haslam (1990) have since been corroborated by some independent investigations. For example, the enhancement of

cAMP accumulation by NO donors, including NP, and the involvement of PDE3 have been documented in human platelets (Andersson and Vinge, 1991; Grunberg *et al.*, 1995; Anfossi *et al.*, 1995; Fisch *et al.*, 1995). However, a few studies have failed to detect an enhanced cAMP accumulation in platelets when cGMP is introduced. Alheid *et al.* (1989) found no evidence of a synergistic interaction between prostacyclin and NP, and claimed that these cyclic nucleotides exert inhibitory effects on platelet aggregation through completely separate mechanisms. Radziszewski *et al.* (1990) reported that although iloprost (a stable analog of PGI_2) and NP synergistically inhibit platelet aggregation, NP did not significantly increase cAMP levels either in the presence or the absence of iloprost. Finally, Beisegel and Grodzinska (1992) failed to detect any effect of SIN-1 (a NO donor) on platelet cAMP levels either in the presence or the absence of iloprost.

However, in the case of vascular smooth muscle, which contains PDE3, synergistic increases in cAMP accumulation were also seen when adenylyl cyclase and guanylyl cyclase were both stimulated (Maurice and Haslam, 1990b; Miyata *et al.*, 1992; Jang *et al.*, 1993; Fiscus *et al.*, 1994).

1.3.6.   Effects of platelet cyclic nucleotides

The major intracellular target of cAMP is PKA. cGMP has multiple intracellular targets, including PKG, certain PDE enzymes, and cGMP-gated ion

channels. In this discussion, the structure and targets of cyclic nucleotide-dependent kinases is examined, focusing on their effects on $[Ca^{2+}]_i$, rap 1b and the vasodilator-stimulated phosphoprotein (VASP).

PKA holoenzymes are composed of a homodimer of two regulatory subunits (R), each bound to a catalytic C subunit (reviewed by Houslay and Milligan, 1997). In many tissues, the RI regulatory subunit is found in the cytosol, whereas the RII subunit is associated with membranes and is phosphorylated by the PKA catalytic subunit (reviewed by J. D. Scott, 1991). Two distinct subtypes of regulatory subunits, α and β, have been identified for both RI and RII (reviewed by Francis and Corbin, 1994). At the amino terminus of the regulatory subunits is found the homodimerization domain which binds two R subunits together, and a pseudosubstrate region which inhibits the activity of the catalytic subunit. The cAMP-dependence of the activity of PKA is caused by the regulatory subunit, which contains two tandem repeat cAMP binding domains: site A (fast-dissociating) and site B (slow-dissociating) (Francis and Corbin, 1994). A single molecule of cAMP may bind to either site, and by doing so causes a conformational change in PKA which "primes" the enzyme for further stimulation by cAMP. Binding of four molecules of cAMP to the holoenzyme leads to dissociation of the catalytic subunits from the regulatory homodimer, thereby relieving the inhibition exerted by the pseudosubstrate region (Francis and Corbin, 1994).

Three mammalian types of catalytic subunit have been described: α and β are

ubiquitously expressed, while the expression of γ appears to be restricted to testes (Scott, 1991). PKA is a member of the AGC kinase subfamily, and thus shares a similar catalytic domain structure and substrate recognition sequence with PKG and PKC, the other two members of this family (Scott, 1991). There are several functional regions within the 250 amino acid catalytic core, responsible for ATP binding, substrate recognition, and regulatory subunit binding. PKA is a serine/threonine kinase, and its recognition sequence is defined by two basic amino acids located one or two residues away from serine or threonine (Scott, 1991).

Two types of PKG have been described; PKG I is widely expressed, and two splice variants (α and β) have been described, whereas PKG II is only expressed in brain, intestine, and kidney (Lohmann *et al.*, 1997). Like PKA, these enzymes are composed of 2 regulatory and 2 catalytic elements, but in the case of PKG, the regulatory and catalytic domains are contiguous on each of two subunits of a homodimeric enzyme (Francis and Corbin, 1994). Upon binding of two molecules of cGMP, PKG undergoes a conformational change which results in 50% of the full activity of the enzyme. Full activity is reached when 4 molecules of cGMP are bound (Scott, 1991).

The selectivity of activation of PKA and PKG by cyclic nucleotides is maintained by a single amino acid residue in the cyclic nucleotide binding pockets of each kinase (Jiang *et al.*, 1992). In PKA this residue is alanine, whereas in PKG it is threonine. When either cyclic nucleotide is present at high concentration, it can cross-

activate the other kinase. For example, high concentrations of cAMP relax smooth muscle by activating PKG (Jiang *et al.*, 1992)

By photoaffinity labelling of PKA with cAMP analogs, the presence of PKA RI subunits in the particulate fraction and of RII subunits in the cytosolic fraction has been demonstrated in human platelets (Haslam *et al.*, 1980; Salama and Haslam, 1984). In these studies, the abundance of RI subunits was fourfold greater than observed for RII, but the level of activity associated with each isoenzyme was approximately equal, suggesting that a large proportion of the RI population is not coupled to catalytic subunits (Salama and Haslam, 1984). PKA and PKG have also both been detected in human platelets in Western blots using rabbit antisera (Eigenthaler *et al.*, 1992).

To identify substrates of PKA and PKG, platelets were initially pre-incubated with $[^{32}P]P_i$ and, after treatment with PGE_1 or NP, ^{32}P-labelled proteins were separated by SDS-PAGE (Haslam *et al.*, 1980). Four targets of PKA were identified using this technique and designated P50, P36, P24, and P22. This list has since been extended by other investigators to include 240, 130 and 42 kDa substrates (Waldmann *et al.*, 1986). Similarly, the targets of PKG action were initially named P50 and P49, and a 130 kDa substrate was recognized later (Haslam *et al.*, 1980; Waldmann *et al.*, 1986). The identities of some of these substrates have since been determined.

Phosphorylation of the membrane-bound P22 or P24 by PKA was initially associated with an increased rate of uptake of Ca^{2+} from the cytosol into the dense tubular

system (Haslam *et al.*, 1979); this is one of the many ways in which cyclic-nucleotide-dependent protein kinases may modulate cytosolic Ca^{2+} levels. Most events associated with platelet stimulation are connected to an increase in cytosolic Ca^{2+}; PKC and $cPLA_2$ are stimulated by Ca^{2+}, MLCK is activated by Ca^{2+}-CaM and is involved in the shape change response, and secretion of the platelet granules is promoted by a rise in Ca^{2+} (reviewed by Haynes, 1993). $[Ca^{2+}]_i$ is tightly regulated by the combined activity of receptor-operated channels, Na^+/Ca^{2+} exchangers (plasma membrane only), ATP-dependent Ca^{2+} pumps, and passive leaks in the plasma membrane and the dense tubular system (Haynes, 1993). Many of the inhibitory effects of cyclic nucleotides are accomplished by lowering $[Ca^{2+}]_i$, and this is predominantly due to the activities of PKA and PKG (Haynes, 1993). PKA promotes uptake of Ca^{2+} into the dense tubular system and also inhibits $InsP_3$-induced Ca^{2+} release from this pool (Haynes, 1993). It has also been claimed that PKA and PKG promote a net efflux of Ca^{2+} out of platelets by increasing the activity of the plasma membrane Ca^{2+}-ATPase, although this is disputed (Haynes, 1993). Furthermore, when internal Ca^{2+} stores are emptied a Ca^{2+} influx through the Ca^{2+}-release activated channel is normally observed; this is also attenuated by PKA and PKG (Doni *et al.*, 1994; Nakamura *et al.*, 1995). Indirect effects include an inhibition of PLC activation (Siess, 1989). The net result of the multiple actions of PKA and PKG is a lowering of $[Ca^{2+}]_i$ by decreasing Ca^{2+} influx into platelets, by enhancing the uptake of Ca^{2+} into the dense tubular store and by making this store more difficult to

release (Haynes, 1993).

The 22 kDa target of PKA has been identified as rap 1b (Siess *et al.*, 1990). Activation of PKA by cAMP elevating agents leads to an increased phosphorylation of rap 1b (Grunberg *et al.*, 1995). When cGMP was also present, platelet cAMP and the phosphorylation of rap 1b were both increased synergistically. However, phosphorylation was only observed after several minutes and was therefore far too slow to be involved in the inhibition of platelet responses (Siess and Grunberg, 1993). The function of rap 1b is not yet understood, but one group has claimed that it is involved in targeting PLCγ1 to the plasma membrane (Torti and Lapetina, 1994).

The 49/50 kDa targets of PKA and PKG have also been identified as vasodilator-stimulated phosphoprotein (VASP) (Eigenthaler *et al.*, 1992). Phosphorylation of VASP is temporally correlated with inhibition of platelet activation (Eigenthaler *et al.*, 1992). Activation of PKA or PKG *in vitro* causes phosphorylation of VASP at three sites, resulting in a change in its mobility on SDS-PAGE (from 46 to 49 or 50 kDa) (Butt *et al.*, 1994). VASP is localized at sites of focal contacts, where transmembrane integrins make contact with the cytoskeleton (Butt *et al.*, 1994). A recent study found that in intact platelets, PKA and PKG convert 100 and 50% of VASP, respectively, to its 50 kDa form, and noted that this ratio of activities is the same as the ratio of the abilities of cAMP- and cGMP-elevating agents to block the expression of the active form of the fibrinogen receptor (Butt *et al.*, 1994). The mechanism underlying the

exposure of the active form of this receptor is one the great unanswered questions in platelet signal transduction, and a tentative link between VASP and inhibition of the expression fibrinogen receptor is indeed intriguing.

1.4.   Cyclic 3', 5'-nucleotide phosphodiesterases (PDEs)

Cyclic nucleotides are key regulators of platelet function. Through the actions of their dependent kinases, they block the responses of platelets to agonists. The cytosolic levels of cyclic nucleotides are steady-state concentrations, because they are a balance between the rates of formation by the respective cyclases and the rates of hydrolysis by PDEs (Ashby, 1990a). Increases in cAMP and cGMP are observed when PDEs are inhibited; conversely, their activities prevent the accumulation of cyclic nucleotides and thereby attenuates the inhibitory signals that the cyclic nucleotides transmit.

There are seven families of PDE genes, which may be further divided into subfamilies and their splice variants (reviewed by Beavo *et al.*, 1994). These families are distinguished by their respective substrate selectivities, modes of regulation, and sensitivities to pharmacological inhibitors. Most of the PDEs isolated to date are homodimeric or multimeric enzymes. PDEs maintain a highly conserved carboxyl terminal catalytic domain in which the amino acid sequence is 25-40% identical among all PDEs and greater than 70% identical within some gene families (Charbonneau *et al.*,

1986). Amino terminal regions comprise the regulatory domains of these enzymes, and consequently the sequences here are dissimilar in different gene families (reviewed by Beavo, 1995).

The expression of each PDE family varies amongst different tissues, resulting in distinct PDE profiles among diverse tissues (Manganiello et al., 1995). Within the cell, the regulation of cyclic nucleotides is a complex integration of the activities of multiple isoenzymes, which differ in their modes of activation, their affinities, their V_{max} values for each cyclic nucleotide, and their subcellular localizations (reviewed by Houslay and Milligan, 1997). Specific manipulation of cellular function by inhibition of the PDEs that are expressed in specific cells is a major goal of the pharmaceutical industry.

Three genes (and seven splice variants) encoding members of the PDE1 family (the Ca^{2+}-CaM activated PDEs) have been identified: PDE1A is found in cardiac and in brain tissues, PDE1B has been discovered in brain, adrenal medulla and kidney tissues, and PDE1C is found in testis and brain (Manganiello et al., 1995; Beltman et al., 1995). Although all members of the family hydrolyse cAMP and cGMP, the K_m of each PDE1 for cGMP varies; that of PDE1A and PDE1B is greater than that of PDE1C (Manganiello et al., 1995). Binding of Ca^{2+}-CaM to the enzyme increases activity by increasing V_{max} and causing a small reduction in K_m for either substrate (Beltman et al., 1995). In vitro phosphorylation of PDE1 by PKA and by CaM-dependent kinases has

been demonstrated, and decreases the affinity of the PDE1 for CaM (Beltman *et al.*, 1995).

PDE2 hydrolyses both cAMP and cGMP. The distinguishing property of this enzyme is that hydrolysis of low concentrations of cAMP is stimulated by the binding of cGMP to a regulatory site (Russell *et al.*, 1973). This enzyme is found in the platelet, and is the focus of this thesis. Its properties and inhibition by EHNA are described in detail in Section 1.5..

PDE3 is also found in human platelets. This enzyme has a low K_m for cAMP, and its activity is inhibited by cGMP (Degerman *et al.*, 1997). Many pharmacological inhibitors of this enzyme have been described; in the present research lixazinone is employed. The characteristics of PDE3 and its inhibition by lixazinone are described in Section 1.6..

PDE4 isoenzymes are selective for cAMP (Manganiello *et al.*, 1995). These enzymes hydrolyze cAMP with a relatively low K_m (3-4 µM), and are strongly inhibited by rolipram and Ro 20-1724 (Manganiello *et al.*, 1995; Thompson, 1991). More isoforms of PDE4 have been cloned than any other PDE family; 4 distinct genes have been reported, and there are at least 15 splice variants (Houslay and Milligan, 1997). The activity of PDE4 enzymes is controlled by both transcriptional regulation and by phosphorylation by PKA (Beavo, 1995). A regulatory role for this enzyme has been described in Sertoli cells, brain, and lung lymphocytes (Beavo, 1995).

Table 1: Properties of phosphodiesterase families

The known properties of each PDE gene family are summarized, including the respective

substrate selectivity for each family. Selective inhibitors, if any are known, are also

listed. The asterisks denote those PDEs that are present in human platelets.

	Family	Substrates	Inhibitors
	1. Ca^{2+}/CaM-stimulated PDEs	cGMP > cAMP	Vinpocetine
*	2. cGMP-stimulated PDEs	cAMP ≈ cGMP	EHNA
*	3. cGMP-inhibited PDEs	cAMP » cGMP	Lixazinone
	4. cAMP-specific PDEs	cAMP	Rolipram
*	5. cGMP-specific PDEs	cGMP » cAMP	Zaprinast
	6. Photoreceptor PDEs	cGMP	None known
	7. HCP1-PDE	cAMP	None known

PDE5 is the cGMP-binding, cGMP-selective phosphodiesterase, and is the third PDE found in platelets (PDE2 and PDE3 are the other two). The activity of this enzyme is potently inhibited by zaprinast. PDE5 is described in fuller detail in Section 1.7..

PDE6 is the cGMP-specific phosphodiesterase found in photoreceptor cells (Beavo, 1995; Beavo et al, 1994; Pyne et al., 1996). Its subunit structure and mode of regulation are rather unusual. PDE6 is a heterotetrameric molecule composed of 2 catalytic subunits (α, β) and 2 inhibitory subunits (γ) (Beavo, 1995; Beavo et al, 1994; Pyne et al., 1996; Manganiello et al., 1995). When light reaches a photoreceptor cell, an isomerization in rhodopsin leads to the exchange of bound GDP for GTP on transducin (T), a heterotrimeric G protein. Tα-GTP dissociates from T$\beta\gamma$, and releases PDE6γ, resulting in activation of PDE6 (Beavo, 1995; Beavo et al, 1994; Pyne et al., 1996; Manganiello et al., 1995). PDE6 hydrolyses local cGMP, which reduces cGMP binding to cGMP-gated cation channels in the plasma membrane (Beavo, 1995). Thus, PDE6 isoenzymes are directly involved in phototransduction in retinal rods and cones.

PDE7 is the most recently described family of PDEs. Little information has been uncovered concerning the regulation and function of this PDE family. mRNA encoding PDE7 has been detected in skeletal muscle and T cells (Beavo et al., 1994). The enzyme binds cAMP with high affinity, but is not susceptible to the same inhibitors as are other families of cAMP phosphodiesterases (Beavo, 1995).

1.5.   cGMP-stimulated phosphodiesterase (PDE2)

1.5.1.   Structure and localization of PDE2

A cGMP-stimulated, cAMP phosphodiesterase activity was discovered by

Sutherland and his colleagues in 1971 (Beavo *et al.*, 1971) and has since been purified

from numerous tissues, including brain (rabbit and bovine), adrenal (bovine), heart

(bovine), and liver (murine and bovine) (Whalin *et al.*, 1988; Murashima *et al.*, 1990;

Martins *et al.*, 1982; Yamamoto *et al.*, 1983). PDE2 has also been purified from human

platelets using a cGMP-affinity chromatography strategy (Grant *et al.*, 1990).

The apparent molecular mass of the purified bovine heart enzyme on SDS-

PAGE was between 105 and 107 kDa. However, when this enzyme was electrophoresed

on native polyacrylamide gradient gels a value of 240 kDa was obtained, suggesting that

native PDE2 is a dimeric molecule (Martins *et al.*, 1982).

To determine the amino acid sequence of PDE2, purified bovine heart cytosolic

PDE2 was cleaved by CNBr and limited proteolysis to produce overlapping peptide

fragments which were subsequently sequenced (Trong *et al.*, 1990). Based on the

peptide sequence obtained, PDE2 comprises 921 amino acids with a calculated molecular

mass of 103 kDa, a value which is consistent with the observed SDS-PAGE mobility of

the monomer (102-107 kDa) (Trong *et al.*, 1990).

At the carboxyl terminus of PDE2 is a region of 259 residues which is

homologous with the catalytic domains of other PDEs (23-35% amino acid identity)

(Trong *et al.*, 1990). Limited proteolysis of PDE2 generated a 36 kDa fragment which included the entire conserved carboxyl terminus region and maintained catalytic activity (Stroop *et al.*, 1989), which demonstrates that this conserved region comprises the catalytic domain (Trong *et al.*, 1990).

Two tandem repeats were discovered towards the middle of the PDE2 molecule. Because these repeats bear some sequence similarity to the cGMP-binding regions of rod and cone PDEs, it was suspected that they formed the noncatalytic cGMP-binding sites of PDE2 (Trong *et al.*, 1990). This suggestion was supported by earlier photolabelling studies that had demonstrated that the noncatalytic cGMP-binding sites are located towards the middle of the molecule (Stroop *et al.*, 1989). Therefore, it was proposed that the structure of PDE2 is that of a chimeric molecule, in which cGMP binds to regulatory sites in the middle of the enzyme, and the catalytic domain is located at the carboxyl terminus (Trong *et al.*, 1990).

The cDNA sequence of PDE2 was determined by a strategy which included screening an adrenal cortex library with two degenerate oligonucleotide probes coding for segments of the bovine heart PDE2 peptide sequence (Sonnenburg *et al.*, 1991). mRNA which hybridized to PDE2 probes was detected in many bovine tissues including various regions of the brain, kidney, lung, spleen and T-lymphocytes (Sonnenburg *et al.*, 1991). A difference was noted in the protection of a 452-base riboprobe directed at the amino terminus; the probe was completely protected from RNase degradation by PDE2 mRNA

from many tissues, but in the brain the probe was only partially protected, suggesting that the mRNA for this enzyme is differentially spliced at the amino terminus (Sonnenburg *et al.*, 1991). In 1994, another group of investigators cloned the cDNA for PDE2 from the particulate fraction of rat brain, and indeed, although most of the cDNA sequence is identical with that cloned from the adrenal cortex, the rat brain cDNA has a small extension at the amino terminus (Yang *et al.*, 1994). Because the extension is highly hydrophobic, the authors proposed that it may be responsible for the membrane association of the enzyme (Sonnenburg *et al.*, 1991).

Rosman *et al.* (1997) have recently identified a third splice variant of the PDE2A gene. This cDNA contains an amino-terminal sequence which is distinct from that of the bovine heart (PDE2A1) and rat brain (PDE2A2) cDNAs. This difference may be functionally significant, because it is thought that amino-terminal sequences are involved in directing PDE2 towards the membrane or cytosol. Northern blots have detected PDE2A3 mRNA in brain, liver, kidney, skeletal muscle, and to a lesser extent in lung and pancreas (Rosman *et al.*, 1997). Platelets were not tested.

1.5.2.   The regulatory cGMP-binding site of PDE2

The localization of the cGMP-binding sites and their mode of activating cAMP phosphodiesterase activity has been examined using limited proteolysis, photolabelling and nucleotide analogue binding studies. Limited chymotryptic digestion of [^{32}P]cGMP-

photolabelled PDE2 generates two major labelled fragments: a 36 kDa fragment which maintains catalytic activity, and a 60 kDa fragment which does not (Stroop *et al.*, 1989). By cleaving the 60 kDa fragment with CNBr, a 28 kDa segment was isolated that comprised the noncatalytic cGMP binding site (Stroop *et al.*, 1989). Binding of cGMP to PDE2 in this region (in the presence of ammonium sulfate) indicates that 2 moles of cGMP bind per mole of homodimer (Stroop *et al.*, 1991). The peptide sequence of the noncatalytic (regulatory) cGMP binding site bore no significant homology to the cAMP-binding domain in the regulatory subunit of cAMP-dependent protein kinase (Charbonneau *et al.*, 1990).

To explore the nature of binding to the noncatalytic site, activators and inhibitors of PDE2 activity were compared with respect to their abilities to release bound [^{3}H]cGMP from the noncatalytic sites (Miot *et al.*, 1985). The analogs that activated cAMP phosphodiesterase activity also potently released [^{3}H]cGMP from the noncatalytic site, whereas those that were inhibitors of PDE activity did not (Miot *et al.*, 1985). This suggested that the noncatalytic cGMP-binding site regulates the cAMP phosphodiesterase activity of PDE2 (Miot *et al.*, 1985; Erneux *et al.*, 1985)

Structure-activity relationships between cGMP and the regulatory binding site were explored in detail using cGMP analogs in which the axial or equatorial exocyclic oxygen atom of the ribose ring was substituted with a sulphur (Erneux *et al.*, 1985). Both the axial and equatorial analogs were similarly poor activators of cAMP

phosphodiesterase activity, implying that at the regulatory site there is an interaction involving negative charge distributed between both exocyclic oxygen atoms (Erneux *et al.*, 1985). Evaluation of analogs which were derivatized at the 5' oxygen of the ribose ring or at the heterocyclic N-7 of cGMP demonstrated that these functional groups are involved in binding to the regulatory site (Erneux *et al.*, 1985).

Evidence for a conformational change during activation of PDE2 by cGMP was sought by testing the sensitivities of basal and activated PDE2 to protease digestion and chemical modification (Stroop *et al.*, 1991). Exposure of new chymotrypsin cleavage sites after activation of PDE2 suggested a conformational change (Stroop *et al.*, 1991). Likewise, only the activated form of PDE2 could be modified by dicyclohexyl-carbodiimide (Stroop *et al.*, 1991).

1.5.3   PDE2 kinetics

PDE2 activity was first noted in rat liver supernatant (Beavo *et al.*, 1970) and has since been partially or highly purified from a wide range of tissues, enabling kinetic studies to be performed. At low concentrations of substrate, the enzyme is more active with respect to cGMP than to cAMP (apparent K_m values 11-16 and 28-36 µM, respectively) (Yamamoto *et al.*, 1983; Whalin *et al.*, 1988). The maximum activity of PDE2 is similar with respect to either substrate, and ranges from 120 - 200 µmol/min/mg (Yamamoto *et al.*, 1983; Whalin *et al.*, 1988). The kinetics of PDE2 activity with respect

to cAMP or cGMP are positively cooperative (Russell *et al.*, 1973). Cooperativity is observed at lower concentrations of cGMP than of cAMP, and the degree of cooperativity is higher for cAMP substrate (Hill coefficients of 1.3 and 1.9, respectively) (Manganiello *et al.*, 1990).

The hallmark of PDE2 activity is that low concentrations of cGMP potentiate the cAMP phosphodiesterase activity of the enzyme. At cGMP concentrations up to 1 μM - in which binding is primarily at the high affinity, regulatory cGMP-binding sites - the cAMP phosphodiesterase activity of PDE2 is increased, compared to that in the absence of cGMP (Russell *et al.*, 1973). This transforms the plot of reaction velocity vs. substrate concentration from sigmoidal to linear (Manganiello *et al.*, 1990). Activation by cGMP decreases the K_m value of PDE2 for cAMP but does not increase the V_{max} (Manganiello *et al.*, 1990).

Similar effects on PDE2 (lowering of the K_m value for cAMP and activation of cAMP hydrolysis) can be induced in the absence of cGMP when the enzyme is incubated at pH 9.5 or 10 in the presence of $MgCl_2$ (Wada *et al.*, 1987). As discussed earlier, the induction by cGMP of altered PDE2 sensitivity to chymotryptic cleavage has been attributed to an allosteric conformational change induced by cGMP binding to the regulatory site (Stroop *et al*, 1991). Whether high pH plus Mg^{2+} causes a conformational change like that induced by cGMP has not been clarified.

When the concentration of [^{3}H]cGMP substrate is too low to saturate the

regulatory binding sites (2 - 40 nM), its hydrolysis at the active site is stimulated by high

concentrations of cAMP (20 μM), indicating that cAMP is also able to bind to the

regulatory site, albeit with much lower affinity than cGMP (Manganiello *et al.*, 1990).

Furthermore, cAMP is able to compete for binding of [^{3}H]cGMP to the regulatory site of

PDE2 (50% of [^{3}H]cGMP is displaced from the regulatory site by 6 μM cAMP) (Erneux

et al., 1985). Taken together, these observations indicate that stimulatory binding

through the regulatory site of PDE2 is not exclusively mediated by cGMP (Manganiello

et al., 1990).

1.5.4. Regulatory function of PDE2

A role for PDE2 in the regulation of cyclic nucleotide levels has been reported

in diverse signalling systems. Before the recognition of EHNA as an inhibitor of PDE2,

the only indications of the regulatory role of this enzyme were the observations that, in

some systems, inhibitory effects on cAMP accumulation were stimulated by cGMP. In

this section, studies that were performed without EHNA and indicate a regulatory role for

PDE2 are described.

In the adrenal cortex, PDE2 is abundant in the zona glomerulosa, the region

which synthesises aldosterone (MacFarland *et al.*, 1991). It is well known that agonists

such as adrenocorticotropic hormone stimulate steroidogenesis in these cells by

increasing cAMP levels, and that atrial natriuretic peptide (ANP) opposes this effect

(MacFarland *et al.*, 1991). Two mechanisms for the action of ANP were proposed: activation of guanylyl cyclase by binding of ANP to its receptor stimulates PDE2 resulting in a reduction in cAMP levels, or the ANP receptor is coupled to G_i which reduces cAMP levels by inhibiting adenylyl cyclase (MacFarland *et al.*, 1991). When the effects of a series of analogs of cAMP were tested, the relative ability of ANP to block the effects of the analogs on steroidogenesis was parallelled by the ability of PDE2 to hydrolyse the analogs (MacFarland *et al.*, 1991). Furthermore, blocking G_i with pertussis toxin did not change the effects of ANP on aldosterone production (MacFarland *et al.*, 1991). These observations are consistent with a model in which the inhibitory effects of ANP on aldosterone production are mediated by activation of PDE2.

A similar observation has been made in PC12 cells, in which increases in cAMP (caused by stimulating adenylyl cyclase through the adenosine receptor) are attenuated by agents which increase cGMP (such as NP or ANP) (Whalin *et al.*, 1991). These effects were prevented by blocking the activity of PDE2 with trequinsin (Whalin *et al.*, 1991). Trequinsin is a non-specific inhibitor of the enzyme, but in these cells PDE2 is the predominant PDE activity (Whalin *et al.*, 1991).

Five PDE isoforms are found in NB-OK-1 cells (PDE1 through PDE5), which makes it possible to study complex interactions between PDEs in regulating cyclic nucleotides (Delporte *et al.*, 1996). The effects of increasing cGMP levels on agonist-induced increases in cAMP were studied in this cell line (Delporte *et al.*, 1996).

Increases in cGMP concentrations (caused by stimulation of guanylyl cyclase by atrial natriuretic factor) were clearly associated with a reduction in cAMP accumulation (when adenylyl cyclase was stimulated) (Delporte *et al.*, 1996). This observation is consistent with a model in which increases in cGMP activate PDE2, resulting in a reduction in cAMP levels (Delporte *et al.*, 1996).

1.5.5.   Effects of EHNA on PDE2-regulated systems

EHNA was first developed as one of a series of adenosine deaminase (ADA) inhibitors, in which the length of a 9-substituted hydrophobic chain on the adenine ring and the chirality at two centres in these 9-substituents were varied (Schaeffer and Schwender, 1974). EHNA was the most potent ADA inhibitor of the series. The authors proposed that EHNA induces a conformational change in ADA, because inhibition was stereoselective (*erythro-* derivatives were much more effective than *threo-* derivatives), and the change in free energy upon EHNA binding was much larger than would be expected simply on the basis of hydrophobic interactions (Schaeffer and Schwender, 1974). The structure of EHNA is depicted in Section 1.8..

The effects of chirality at positions 2′ and 3′ in the 9-substituted adenine derivatives have also been examined by testing the (+) and (-) stereoisomers of EHNA and THNA (the *threo-* analog) (Bessodes *et al*, 1982). The degree of inhibition of ADA activity was quite different between the chiral isomers; (+)-2S, 3R-EHNA was by far the

most potent inhibitory form (K_i, 2 nM). (+) EHNA is typically sold in a racemic mixture with the relatively weak (-) chiral isomer (K_i, 500 nM); the mixture is a strong inhibitor of purified human ADA (K_i, 4 nM) (Bessodes *et al*, 1982).

In 1995, EHNA was recognized as not only an ADA inhibitor but also as a selective inhibitor of PDE2 in porcine (IC_{50}, 2 μM) and human (IC_{50}, 0.8 μM) myocardium (Podzuweit *et al.*, 1995). Because EHNA can inhibit both ADA and PDE2, this group suggested that the active and/or regulatory sites of these enzymes may be similar (Podzuweit *et al.*, 1995). The mechanism of inhibition of PDE2 by EHNA has not yet been determined. However, some details of its action on ADA have been uncovered (Frieden *et al.*, 1980). The two-step reaction begins with a fast binding step, which is followed by a rearrangement of the EHNA-ADA complex.

Involvement of PDE2 in the regulation of the Ca^{2+} current in cardiac myocytes has been specifically demonstrated using EHNA. In these cells, increases in cAMP activate cAMP-dependent protein kinase, and phosphorylation by this enzyme leads to activation of L-type Ca^{2+} channels. The cAMP-stimulated Ca^{2+} current was only increased by inhibition of PDE3 or PDE4, but not by inhibition of PDE2 (Méry *et al.*, 1995). The cAMP-stimulated Ca^{2+} current was opposed by agents which increase cGMP, such as nitroprusside or SIN-1, and this effect could be blocked only by inhibiting PDE2 (Méry *et al.*, 1995). These observations suggest that in the absence of cGMP, PDE2 is inactive and any regulation of cAMP by PDEs is attributable to PDE3 or PDE4 (Méry *et*

al., 1995). However, when cGMP was directly introduced into cells or its synthesis was stimulated by NO donors, activation of PDE2 inhibited the L-type Ca^{2+} channel activity, presumably by reducing activation of cAMP-dependent protein kinase (Méry *et al.*, 1995).

The effects of EHNA on PDE2 have also been studied in the perfused rat lung, although the involvement of PDE2 was recognized accidentally (Haynes *et al.*, 1996). Vasoconstriction is observed in the pulmonary vasculature in response to low oxygen levels. At the same time, hypoxic tissues accumulate extracellular adenosine, which favours vasodilation by increasing cAMP levels. The investigators used EHNA to inhibit ADA (and thus prevent the breakdown of adenosine), and it was found that although EHNA blocked the hypoxic pressor response, this was due to its action on PDE2 rather than on ADA (Haynes *et al.*, 1996). A role for PDE2 in the regulation of the pressor response was established, although it was not clear whether the substrate of PDE2 was cGMP or cAMP (Haynes *et al.*, 1996).

1.5.6.   Platelet PDE2

PDE2 has been purified from human platelets using a cGMP-affinity chromatography strategy (Grant *et al.*, 1990). The molecular mass of the platelet enzyme, 105 kDa, was similar to that of the bovine heart enzyme (Grant *et al.*, 1990). The kinetic properties of platelet PDE2 were also similar to those observed with enzyme

from other sources; i.e. comparable V_{max} values for hydrolysis of cAMP or cGMP, a lower K_m for cGMP than for cAMP (35 and 50 µM, respectively), and a potentiation of cAMP hydrolysis by low levels of cGMP were also seen (Grant *et al.*, 1990).

1.6. cGMP-inhibited phosphodiesterase (PDE3)

cGMP-inhibited phosphodiesterase (PDE3) has been the most actively investigated PDE in platelets and vascular tissues. Inhibition of the enzyme increases cAMP, which leads to inhibition of aggregation, increased cardiac contractile force and reduced blood pressure (Alvarez *et al.*, 1986; Murray and England, 1992; Nicholson *et al.*, 1991). Due to the potential therapeutic effects of inhibiting this enzyme, much research has been devoted to understanding the biochemistry of PDE3 and to developing specific inhibitors.

When human platelet PDEs were first separated by DEAE-cellulose chromatography in 1976, a low K_m cAMP phosphodiesterase activity was noted (Hidaka and Asano, 1976). A 61 kDa protein with a low K_m for cAMP was purified from platelets (Grant and Colman, 1984), but was later discovered to be a proteolytic fragment of PDE3 (MacPhee *et al.*, 1986). Recently, intact platelet PDE3 was purified using an affinity chromatography strategy, though fragments were still present (Degerman *et al.*, 1994). The native enzyme is a homodimer, composed of 110 kDa subunits (Degerman *et al.*, 1994). PDE3 activity is observed with very low concentrations of either cAMP or cGMP

(K_m 0.1 to 0.8 µM) (Degerman *et al.*, 1994). However, the reaction velocity is very low

when cGMP is the substrate (0.9 µmol/min/mg compared to 6.1 µmol/min/mg for

cAMP), so that cGMP competitively inhibits PDE3 activity (Degerman *et al.*, 1994;

Degerman *et al.*, 1997).

Two genes for PDE3 have been described; PDE3A is found in platelets, heart,

and vascular smooth muscle, and PDE3B is found in adipocytes, hepatocytes and many

other tissues (Degerman *et al.*, 1997). PDE3 activity has been observed in particulate or

soluble fractions, and in the platelet the activity is predominantly soluble (Degerman *et

al.*, 1995). A hydrophobic region at the amino terminus of the enzyme aids in membrane

association of the particulate form (Le Roy *et al.*, 1996). Phosphorylation of a PKA

consensus site in the amino terminal region activates the enzyme (Degerman *et al.*, 1997).

There has been a great effort by the pharmaceutical industry to develop

inhibitors of platelet PDE3; these have demonstrated broad-spectrum inhibition of

platelet responses, although some complications have been reported (Meanwell, 1991).

Inhibitors of PDE3 typically include two major structural features: a phenyl group near to

a polarizable group with an adjacent hydrogen. The latter group is usually part of a 5 or 6

membered ring structure (Erhardt and Chou, 1991; Meanwell, 1991)

Lixazinone is a potent inhibitor of platelet PDE3 (K_i, 0.5 nM) (Alvarez *et al.*,

1986,). Kinetic studies on purified PDE3 have suggested a mixed competitive and

noncompetitive mechanism of inhibition (Alvarez *et al.*, 1986). However, a similar

mixed pattern of inhibition was observed when the C-terminus of PDE3 was recently

expressed, but not with the intact enzyme (Tang *et al.*, 1997). It was suggested that the

mixed inhibition observed by Alvarez *et al.* (1986) was due to proteolysis of the enzyme.

The structure of lixazinone is depicted in Section 1.8.. This compound is composed of a

planar tricyclic ring system which is coupled through an ether linkage to a bulky

lipophilic side chain; this latter moiety is likely to interact with a bulk-tolerant

hydrophobic pocket in the enzyme (Alvarez *et al*, 1986; Venuti *et al.*, 1988).

1.7.   cGMP-binding, cGMP-selective phosphodiesterase (PDE5)

The cGMP-binding, cGMP-selective phosphodiesterase (PDE5) is the only

PDE activity detected in platelets other than PDE2 and PDE3 (Ito *et al.*, 1996). Early

studies were performed on PDE5 isolated from lung tissue, where it is in rich supply.

However, the platelet isoenzyme has also been studied (Hamet and Tremblay, 1988;

Robichon, 1991).

The activity of PDE5 is significantly greater with respect to cGMP than cAMP

(K_m values, 0.22 and 40 μM, and V_{max} values, 2.9 and 1.3 μmol/min/mg, for cGMP and

cAMP, respectively) (Sheth and Colman, 1995a). Platelet PDE5 is a homodimer

composed of 95 kDa subunits (Robichon, 1991). At the amino terminus of the enzyme, a

tandem repeat forms a noncatalytic cGMP binding site homologous to that described for

PDE2 (Beltman *et al.*, 1993, Sheth and Colman, 1995a). Binding of cGMP at this site

causes an electronegative shift in the HPLC elution pattern of the enzyme, suggesting a conformational change (Sheth and Colman, 1995a). The observation that binding of cGMP to the noncatalytic site promotes phosphorylation of PDE5 by cyclic nucleotide-dependent kinases is perhaps linked to this putative conformational change (Beltman *et al.*, 1993; Sheth and Colman, 1995a; Pyne *et al.*,1996). PDE5 is phosphorylated at a single site (towards the amino terminus) by PKA, and at the same site by PKG at a tenfold higher rate (Beltman *et al.*, 1993).

The effects of phosphorylation of the enzyme are not entirely clear. Early studies did not demonstrate any effect on PDE5 activity attributable to phosphorylation (Beltman *et al.*, 1993). However, a more recent analysis has shown that incubation of the guinea pig lung enzyme with the catalytic subunit of PKA results in phosphorylation and markedly increased enzyme activity (Pyne *et al.*, 1996). In light of this finding, a model in which PDE5 is involved in a negative feedback loop involving PDE3 was suggested; agents which increase cGMP will inhibit PDE3 and the resulting cAMP increases will activate PKA, which could then stimulate PDE5 to turn off the cGMP signal and close the feedback loop. However, since both PKA and PKG phosphorylate PDE5 at the same site *in vitro* (Beltman *et al.*, 1993), this simple model is unlikely to be correct.

Emerging studies have suggested that the regulation of PDE5 is more complex than had been initially realized. Because its kinetic and cGMP-binding properties are highly homologous with those of the photoreceptor PDE (PDE6), it was expected that

when the gene for the lung enzyme was cloned, it would prove to be similar to PDE6 (Beavo, 1995). However, the sequence disparities between the lung and photoreceptor genes established that they constitute two separate families, PDE5 and PDE6, respectively (Pyne *et al.*, 1996). Nevertheless, it appears that these two families are indeed similar; thus, although their sequences are different, these two enzymes may be regulated in a similar way (Pyne *et al.*, 1996). Briefly, PDE6 is inhibited by coupling to its γ subunit, and inhibition is relieved when the photoreceptor G protein transducin becomes activated (Pyne *et al.*, 1996). Similarly, airway smooth muscle PDE5 forms complexes with two low-molecular mass proteins (14 and 18 kDa), which are immunologically related to PDE6γ (Pyne *et al.*, 1996). PKA may phosphorylate these small proteins and thereby modify their effects on PDE5 activity (Pyne *et al.*, 1996).

Recently, the cGMP-hydrolysing phosphodiesterase activities of human aorta, lung, kidney, heart and platelets were evaluated (Ito *et al.*, 1996). Platelets were the richest source of PDE5 in all the tissues tested, and in these cells, PDE5 was the major cGMP phosphodiesterase. In this study, inhibition of PDE5 converted a sub-threshold concentration of SIN-1 (which stimulates guanylyl cyclase) into a strong inhibitor of platelet aggregation (Ito *et al.*, 1996).

1.5.   Objectives of this thesis

At the outset of this investigation, very little was known about the role of

PDE2 in human platelets. The main goal of this research, therefore, was to investigate the regulation of platelet cyclic nucleotide levels and platelet aggregation by PDE2.

When this project commenced, the finding that EHNA was an inhibitor of PDE2 had just been published (Podzuweit *et al.*, 1995). EHNA appeared to be a potentially useful compound in studies on platelet PDE2, but the selectivity its inhibitory effects on the PDEs present in platelets had to be assessed first.

Earlier investigations in this laboratory had shown that agents which increase cGMP, such as NP, exert biphasic effects on cAMP accumulation. No explanation for the inhibitory component of the action of NP had been advanced; therefore, the involvement of PDE2 was explored using EHNA. Similarly, it had been noted that a much greater accumulation of cAMP resulted when PDE3 was inhibited by lixazinone than when this enzyme was inhibited by an NO donor (i.e. cGMP). It was possible, therefore, that cGMP was exerting effects on cAMP accumulation additional to the inhibition of PDE3, and an objective of this research was to test whether the additional apparently inhibitory effect of cGMP was mediated by PDE2.

It is well established that PDE3 is the major cAMP phosphodiesterase in resting platelets. However, PDE2 also hydrolyses cAMP and its role in regulating platelet cAMP levels had never been studied. Since PDE2 has a high K_m value for cAMP, a further goal of this research was to investigate whether cAMP is regulated by PDE2 when platelet cAMP levels are high.

1.8.   Structures

Prostacyclin
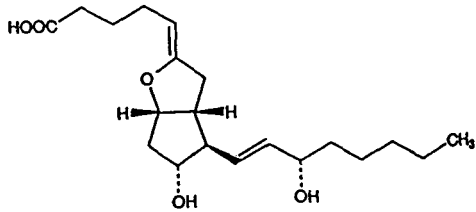
EHNA

Coformycin
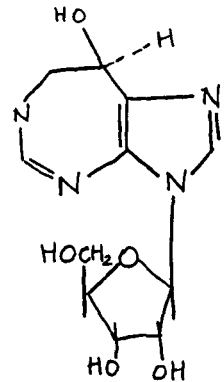
Lixazinone
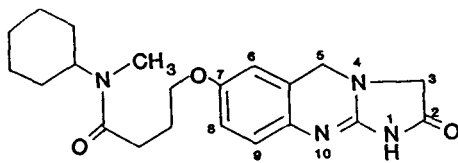
ODQ
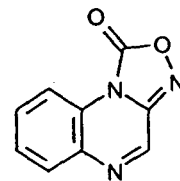
2. EXPERIMENTAL

2.1. Materials

[2,8-^{3}H]Adenine (26.9 Ci/mmol), [2,8-^{3}H]cAMP (27.0 Ci/mmol), [8-^{3}H]cGMP
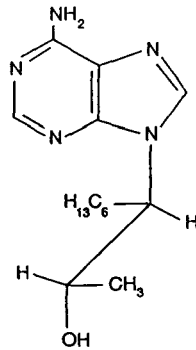
(9.3 Ci/mmol) and [U-^{14}C]AMP (590.4 mCi/mmol) were supplied by NEN Products

(Mississauga, ON, Canada). [8-^{14}C]GMP (50 mCi/mmol) and [8-^{3}H]guanine

(11.9 Ci/mmol) were obtained from Moravek Biochemicals (Brea, CA, U.S.A.) and

[8-^{14}C]ATP (50 mCi/mmol) and [8-^{14}C]cAMP (54 mCi/mmol) were from ICN (Costa

Mesa, CA, U.S.A.). [8-^{14}C]GTP (52 mCi/mmol), [8-^{14}C]cGMP (52 mCi/mmol) and

ACS (aqueous counting scintillant) were from Amersham (Oakville, ON, Canada). All

nucleotides, zaprinast, type I insoluble bovine achilles tendon collagen, pepstatin, PMSF,

nitroprusside (NP), bovine serum albumin (BSA), heparin and neutral alumina (WN-3)

were purchased from Sigma (St. Louis, MO, U.S.A.) and leupeptin from Boehringer-

Mannheim (Laval, PQ, Canada). 1H-[1,2,4]oxadiazolo[4,3-a]quinoxalin-1-one (ODQ)

was from BIOMOL (Plymouth Meeting, PA, U.S.A.) and Hepes was purchased from

BioShop (Burlington, ON, Canada). DEAE-Sephacel was supplied by Pharmacia

Biotech (Montreal, PQ, Canada), Affi-Gel 601 Boronate affinity gel and Dowex-50 resin

(AG 50W-X8, 100-200 mesh, H$^+$ form) by Bio-Rad Laboratories (Mississauga, ON,

Canada). Cellulose powder (MN 300 HR) was from Mandel Scientific (Guelph, ON,

Canada). 2′,5′-Dideoxyadenosine (DDA) was obtained from P-L Biochemicals

(Milwaukee, WI, U.S.A.). Lixazinone (Syntex Corporation, Palo Alto, CA, U.S.A.) and

PGI$_2$ (Upjohn, Kalamazoo, MI, U.S.A.) were gifts. EHNA was generously donated by Dr. T. Podzuweit (Max Planck Institute for Physiological and Clinical Research, Bad Nauheim, Germany), and thrombin by Dr. J. W. Fenton II (New York State Department of Health, Albany, NY, U.S.A.).

2.2.   Partial purification of platelet cyclic nucleotide phosphodiesterases

2.2.1.   Isolation of human platelets

Platelets were isolated by modification of the method of Mustard *et al.* (1972). Blood was taken with informed consent from donors who stated that they had taken no drugs for at least 1 week. Fresh blood (210 ml) was collected into 38 ml of acid-citrate-dextrose anticoagulant (0.085 M trisodium citrate, 0.065 M citric acid, 2% dextrose, final pH of the blood, 6.5) in a siliconized glass flask. Platelet-rich plasma (PRP, approximately 60 ml) was separated from red cells by centrifugation at room temperature (138 g_{av}, 2 x 15 min). Platelets were isolated from PRP by centrifugation (1, 200 g_{av}, 15 min, room temperature), and resuspended in 10 ml of Ca^{2+}-free Tyrode's solution (135 mM NaCl, 2.7 mM KCl, 11.9 mM NaHCO$_3$, 0.42 mM NaH$_2$PO$_4$ and 1 mM MgCl$_2$) containing 5 mM Pipes buffer, pH 6.5, apyrase (90 to 120 µg/ml), BSA (3.5 mg/ml), dextrose (1 mg/ml) and heparin (50 i.u./ml). After incubation of the suspension at 37 °C for 15-20 min, platelets were isolated (800 $g_{av.}$, 10 min, room temperature) and washed a second time in the same medium, except that Ca^{2+} was present (2 mM CaCl$_2$), and

heparin was excluded. The concentration of platelets in the suspension was determined by diluting samples (10 µl) in fixative (1 ml containing 1.5% formaldehyde, 5 mM EDTA, pH 7.4, and 0.154 M NaCl) and counting the platelets after 20 min in a hemocytometer (Reichert Bright Line) with the aid of a phase contrast microscope.

2.2.2. Preparation of platelet supernatant

Platelets were collected by centrifugation (800 $g_{av.}$, 10 min, room temperature) and resuspended at 4 x 10^9 platelets/ml in ice-cold Buffer A (50 mM Tris acetate, adjusted to pH 6.0 with acetic acid, 1 mM EDTA,) containing 4 µM pepstatin, 80 µM chymostatin, 400 µM leupeptin, 1 mM PMSF and 5 mM 2-mercaptoethanol. The suspension was immediately sonicated (Fisher model 300) in a salted ice bath (six times, 15 s each, with a 1 min cooling step between each sonication). Intact platelets and platelet granules were rapidly removed by centrifugation (20, 000 $g_{av.}$, 20 min, 4 °C) and soluble platelet proteins were then isolated (100, 000 $g_{av.}$, 1 h, 4 °C) and chromatographed. Samples (250 µl) of the lysate, 20, 000 $g_{av.}$ supernatant and 100, 000 $g_{av.}$ supernatant were reserved for determination of protein concentrations and PDE activities.

2.2.3. Separation of platelet PDEs by DEAE-Sephacel chromatography

Soluble platelet proteins were chromatographed by modification of the method

of Hidaka and Asano (1976). DEAE-Sephacel (4 ml) was equilibrated in 10 ml of Buffer

B containing 50 mM Tris, adjusted to pH 6.0 with acetic acid, 1 mM EDTA and 0.8 M

sodium acetate (4 °C, overnight). On the following day, the resin was washed and packed

into a column (0.75 cm diameter, 2 ml bed volume) in Buffer A and washed overnight in

the same buffer solution (4 °C, 0.5 ml/h flow rate, Pharmacia P-1 peristaltic pump).

Platelet supernatant (approximately 7 ml, 8 mg of protein) was loaded onto the column

(7 ml/h flow rate) on the day that blood was collected. Unbound platelet proteins were

removed by washing the column with Buffer A (10 ml, 7 ml/h). Bound platelet proteins

were eluted by a linear sodium acetate gradient (0 to 0.8 M, 40 ml total volume, 7 ml/h

flow rate) obtained by mixing Buffers A and B, each containing 1 μM pepstatin, 20 μM

chymostatin, 50 μM leupeptin, 5 mM 2-mercaptoethanol and 0.5 mM PMSF. Fractions

(0.5 ml) were collected using a LB Bromma 2112 fraction collector and alternate

fractions were assayed for PDE activity with 0.5 μM [^{3}H]cAMP, 10 μM [^{3}H]cAMP, and

10 μM [^{3}H]cGMP as substrates.

2.2.4.   SDS-polyacrylamide gel electrophoresis

Samples of chromatography fractions (40 μl) were mixed with 5X Laemmli

buffer (313 mM Tris-HCl, pH 6.8, 50% glycerol, 15% SDS, 25% 2-mercaptoethanol,

0.0125% (w/v) bromophenol blue), heated at 100 °C for 2 min and electrophoresed on

10% polyacrylamide separating gels (70 V overnight, BioRad 3000Xi) (Laemmli, 1970).

Gels were stained with Coomassie Brilliant Blue R solution (1 g/L) in water/methanol/acetic acid (5:5:1, by vol.) for 1 h. Excess dye was removed by soaking in destaining solution (2 x 45 min; water/methanol/acetic acid (5:5:1, by vol.)). Background dye was removed from the gels by soaking overnight in 10% acetic acid solution. After removing acetic acid from the gels by soaking them in distilled water (2 x 45 min), they were dried under vacuum.

2.2.5. Protein assays

Protein was assayed according to the method of Lowry *et al.* (1951), using a mixture of human serum albumin (5 g/dL) and globulin (3 g/dL) as a standard. Protein concentrations were calculated using Quant II software (Beckman).

2.3. Measurements of PDE activity

2.3.1. PDE assays

Cyclic 3',5'-nucleotide phosphodiesterase activities were assayed by a modification of published methods (Davis and Daly, 1979; Reeves *et al*, 1987). The reaction mixture contained, in a final volume of 100 µl, 50 mM Hepes buffer (pH adjusted to 7.5 with KOH), [^{3}H]cAMP or [^{3}H]cGMP (200, 000 dpm total radioactivity, concentration as indicated), 1 mM EGTA, 5 mM $MgCl_2$ and 0.1 mM leupeptin. Assays were initiated by addition of an appropriate volume of enzyme (6 to 20 µl, resulting in

less than 15% hydrolysis of the substrate) to the reaction mixture. Incubations (5 min in a

37 °C water bath) were terminated by addition of EDTA (final concentration 5 mM) to

chelate Mg^{2+} ions and boiling of the samples (2 min). After cooling, samples were mixed

with recovery markers (10 μl of [^{14}C]AMP or [^{14}C]GMP, 2, 000 or 4, 000 dpm,

respectively) and buffer (0.5 ml 50 mM Hepes, adjusted to pH 8.5 with KOH) containing

100 mM $MgCl_2$.

2.3.2.   Boronate affinity chromatography

Nucleotide 5′-monophosphates were isolated by modification of published

methods (Hageman and Kuehn, 1977; Davis and Daly, 1979; Reeves et al, 1987).

Boronate affinity gel was equilibrated with buffer (15 ml, 50 mM Hepes, pH 8.5,

100 mM $MgCl_2$) and packed into columns (0.5 cm in diameter to give a 0.8 ml bed

volume). PDE assay reaction mixtures were centrifuged to remove protein precipitates

(16. 000 g, 4 min, 4 °C) and loaded onto the boronate columns. Unreacted [^{3}H]cAMP or

[^{3}H]cGMP was washed from the column with equilibration buffer (20 ml). Labelled

AMP or GMP was eluted with 250 mM acetic acid into scintillation vials

(2.5 ml acetic acid; the first 0.5 ml was discarded). The boronate columns were

regenerated by washing with 250 mM acetic acid (10 ml), followed by equilibration

buffer (10 ml), and finally stored at 4 °C in buffer (5 ml) until used again. Eluted samples

were counted for ^{3}H and ^{14}C in ACS (10 ml) in a Beckman liquid scintillation counter.

2.3.3. Calculations

The $[^{14}C]AMP$ or $[^{14}C]GMP$ recovered after boronate chromatography was calculated as a percentage of that added. The amounts of $[^3H]AMP$ or $[^3H]GMP$ were then corrected for the recoveries of $[^{14}C]AMP$ or $[^{14}C]GMP$, respectively. The mean ($\bar{x}$) and standard error of the mean (S.E.M.) of triplicate PDE assays, expressed as percentages of the $[^3H]cAMP$ or $[^3H]cGMP$ added to the assay reaction mixture, were calculated according to:

$$\bar{x} = (\sum x) / n \qquad\qquad \text{S.E.M.}_{(\bar{x})} = [\sum(\bar{x} - x)^2 / n - 1]^{\frac{1}{2}} / n^{\frac{1}{2}}$$

The mean percentage cyclic $[^3H]$nucleotide hydrolysis ($\bar{x}$) was corrected by subtracting no-enzyme blank values ($\bar{y}$, approximately 0.3 to 0.6% of $[^3H]cAMP$ and 0.6 to 0.8% of $[^3H]cGMP$) and the error associated with the corrected percentages (the standard error of the difference, S.E.D.) was calculated as:

$$\text{S.E.D.}_{(\bar{x} - \bar{y})} = [(\text{S.E.M.}_{\bar{x}})^2 + (\text{S.E.M.}_{\bar{y}})^2]^{\frac{1}{2}}$$

In some experiments with inhibitors of PDE activity, the mean cyclic nucleotide hydrolysis (b) observed in the presence of the inhibitor was expressed as a percentage of no-inhibitor control values (a). In this case, the standard error of the quotient (S.E.Q.)

was calculated as:

$$S.E.Q. = (100\ \bar{b}/\bar{a})\ [\ (S.E.M._{\bar{b}}/\bar{b})^2 + (S.E.M._{\bar{a}}/\bar{a})^2\ ]^{\frac{1}{2}}$$

2.4.   Analysis of enzyme inhibition

IC_{50} values were calculated using GraFit version 3.0 software (Erithacus

Software Limited, Staines, UK) according to the following formula:

$$b = a\ /\ [\ 1 + (c\ /\ IC_{50})^S\ ]$$

where c is the inhibitor concentration, b is the inhibited enzyme activity, a is the control

enzyme activity, and S is a slope factor.

2.5.   Platelet cyclic nucleotide measurements

2.5.1.   Prelabelling of human platelets

Human platelets were isolated according to the method described for PDE

purification (Section 2.2.1), except that the second resuspension of platelets was in

Tyrode's solution (10 ml) containing 5 mM Hepes (adjusted to pH 7.4 with HCl), apyrase

(135 µg/ml) and BSA (3.5 mg/ml). Platelet metabolic nucleotide pools were labelled

with ^{3}H by modification of the method of Maurice and Haslam (1990). Adenine

nucleotides were labelled by incubating platelet suspension (2.0×10^9 platelets/ml, 37 °C) with 1 µM [^{3}H]adenine (20 Ci/mmol) for 30 min at 37 °C. Platelets were collected by centrifugation (800 g, 10 min, room temperature) and resuspended in the same medium (20 ml, 1×10^9 platelets/ml) containing 1.5 µM [^{3}H]guanine (5 to 10 Ci/mmol). Labelling of the guanine nucleotide pool proceeded for 1 h at 37 °C. Platelets were collected by centrifugation (800 g, 10 min, room temperature) and finally resuspended in the same Tyrode's solution at 4.5×10^8 platelets/ml, except that the concentration of apyrase was reduced (6 µg/ml).

2.5.2. Measurement of changes in platelet cyclic nucleotides

Platelet cyclic nucleotide experiments were performed by a modification of the method of Maurice and Haslam (1990). Standard incubation mixtures contained 450 µl of labelled platelet suspension (2×10^8 platelets) with 50 µl of additions. NP and DDA were dissolved in 0.154 M NaCl. Any additions of PGI$_2$ (dissolved in 0.139 M NaCl containing 9.4 mM Na$_2$CO$_3$) were made 12 s before incubations commenced to obviate breakdown of the compound at physiological pH. EHNA, zaprinast, lixazinone, ODQ or coformycin were dissolved in DMSO (final DMSO concentration not exceeding 0.2% (v/v)). The reaction was initiated by mixing labelled platelet suspension with the additions. Incubations (1 min, 37 °C) were terminated by adding ice-cold trichloroacetic acid (TCA, final concentration 10% (v/v)) and transferring samples to an ice bath. Each

sample was mixed with [^{14}C]cAMP and [^{14}C]cGMP recovery markers (1 000 dpm of each) and stored overnight (4 °C).

2.5.3. Isolation of platelet cAMP and cGMP

Platelet cAMP and cGMP were isolated by the methods of Maurice and Haslam (1990). Cyclic 3',5'-nucleotides were separated from other nucleotides by chromatography on neutral alumina (White and Zenser, 1971). Neutral alumina (1.5 g, Sigma WN-3) was packed into glass columns (1 x 17 cm) and washed with 10% TCA (9 ml). After centrifugation (16, 000 g, 4 min, 4 °C), labelled platelet extracts were applied to the columns. The alumina was freed of unbound components by washing again with 10% TCA (9 ml), followed by distilled water (9 ml). Cyclic 3',5'-nucleotides were eluted with 0.2 M ammonium formate adjusted to pH 6.0 with formic acid (5 ml in total, the first 2 ml were discarded) and the samples were acidified by the addition of 3 M HCl (50 µl, 50 mM final concentration).

cGMP was separated from cAMP by Dowex cation exchange chromatography (Maurice and Haslam, 1990a; Jakobs *et al.*, 1976). Dowex (in a 1:1 slurry in 50 mM HCl) was poured into glass columns (1 x 28 cm, 5 ml bed volume) and washed with 50 mM HCl (5 ml). After loading the acidified alumina eluates (3 ml), the resin was washed again with 50 mM HCl (6 ml). cGMP was eluted by the same solution (8 ml) and collected into scintillation vials. These samples were neutralized by the addition of 8 M

NaOH (50 μl) and 161 mM Hepes (50 μl) prior to lyophilization. After washing the

Dowex resin with distilled water (8 ml), cAMP was eluted into labelled scintillation vials

by a further addition of water (8 ml). All samples were frozen and lyophilized. ^{3}H and

^{14}C in rehydrated samples (0.5 ml) were counted in ACS (8 ml) in a liquid scintillation

counter.

2.5.4.   Measurement of platelet ATP and GTP

[^{14}C]ATP (30 000 dpm) or [^{14}C]GTP (50 000 dpm) was added to control

samples of TCA-treated labelled platelet suspension. After chilling for 30 min,

precipitated platelet proteins were removed by centrifugation (16 000 g, 4 min, 4 °C).

TCA was extracted from the supernatants with water-saturated diethyl ether (3 times

using 3 vol of ether). Samples were stored overnight at -20 °C. Labelled platelet ATP

and GTP were isolated by modification of the cellulose chromatography method of

Maurice and Haslam (1990). A sample of the extract containing labelled ATP (20 μl)

was mixed with unlabelled ATP and GTP (240 nmoles and 120 nmoles, respectively) to

aid in visualization of the nucleotides. A similar procedure was carried out with extract

containing labelled GTP (using 120 nmoles of ATP and 240 nmoles of GTP). Each

mixture was applied to a separate cellulose plate (MN 300 HR) and nucleotides were

resolved using two-dimensional TLC. The plates were developed twice in the first

dimension in

n-butanol/acetone/acetic acid/14.8 M NH_3/H_2O (90:30:20:1:60 by volume, approximately

3.5 h each time) and once in the second dimension in isobutyric acid/14.8 M $NH_3/0.5$ M

$EDTA/H_2O$ (250:10:1:143 by volume, 5-6 h). Nucleotides were visualized with UV light

and scraped from the plates into 1.5 ml microcentrifuge tubes. Nucleotides were

extracted from cellulose by sonication in distilled water (0.5 ml) and centrifugation (16

000 *g*, 2 min, room temperature). The supernatants were counted for ^3H and ^{14}C in ACS

(8 ml) in a liquid scintillation counter.

2.5.5.   Dual label scintillation counting

For both the PDE assays and the measurements of cyclic [^3H]nucleotides in

prelabelled platelets, ^{14}C-labelled recovery markers were added so that the amounts of

^3H-labelled compounds found after column chromatography could be corrected for

losses. A dual label program in the scintillation counter (Beckman LS 5000 TA)

monitored the levels of ^{14}C and ^3H in each sample. Within the program, channel

windows of 1-320 and 450-670 were found to be optimal for ^3H and ^{14}C, respectively.

Each program was first calibrated by preparing a series of chemically quenched standards

in the same cocktail used for the experimental samples. From these standards,

relationships between the H number measured with an internal standard and the counting

efficiencies in each channel were determined and stored in the computer's memory. With

experimental samples, the counts in each channel were corrected automatically for

chemical quench and cross-over to give calculated values for ^{3}H and ^{14}C dpm.

2.5.6. Calculation of results

The uptake of [^{3}H]adenine by platelets during the labelling procedure was estimated by measuring the difference between the total radioactivity of the platelet suspension after addition of [^{3}H]adenine and the ^{3}H that remained in the supernatant after isolation of the platelets. A similar procedure was followed when [^{3}H]guanine uptake was calculated, except that allowance was made for the [^{3}H]adenine already taken up by the platelets. Approximately 40% of [^{3}H]adenine and 75% of [^{3}H]guanine were taken up.

The total platelet [^{3}H]ATP and [^{3}H]GTP were determined by TLC of samples of labelled platelet extracts (Section 2.5.4.). The recovery of the added [^{14}C]ATP and [^{14}C]GTP in TLC eluates was typically 1.0 to 1.5% (the maximum possible was 2%). The values for the [^{3}H]ATP and [^{3}H]GTP extracted after TLC were corrected for the recoveries of [^{14}C]ATP and [^{14}C]GTP, respectively. In this study, 0.5 to 0.7 μCi of [^{3}H]ATP and 1.4 to 1.6 μCi of [^{3}H]GTP were present in each sample of labelled platelets (0.45 ml, 2 x 10^8 platelets).

Platelet [^{3}H]cAMP and [^{3}H]cGMP were isolated by alumina and Dowex 50 chromatography. The recoveries of [^{14}C]cAMP and [^{14}C]cGMP were calculated as percentages of the ^{14}C added to samples before chromatography. The amounts of [^{3}H]cAMP and [^{3}H]cGMP were corrected by the recoveries of [^{14}C]cAMP and

[^{14}C]cGMP, respectively. Corrected [^{3}H]cAMP and [^{3}H]cGMP values were expressed as percentages of total platelet [^{3}H]ATP and [^{3}H]GTP, respectively. The means and S.E.M. from triplicate assays were calculated as described in Section 2.3.3.. In unstimulated platelets, [^{3}H]cAMP levels were typically 0.03 to 0.04% of the platelet [^{3}H]ATP and [^{3}H]cGMP levels were 0.005 to 0.009% of the platelet [^{3}H]GTP %.

2.6. Human platelet aggregation studies

2.6.1. Isolation of human platelets

Platelets were collected and washed as described for PDE purification (Section 2.2.1.), except that the second resuspension of platelets was in Tyrode's solution containing 2 mM CaCl$_2$, 5 mM Hepes buffer, pH 7.40, BSA (3.5 mg/ml), and apyrase (135 μg/ml). Platelets were finally resuspended in the same medium (0.4 to 0.5 x 10^8 platelets/ml), except that the concentration of apyrase was decreased to 13 μg/ml to avoid interference with the aggregation reaction. The platelet suspension was incubated at 37 °C for at least 20 min before each experiment began, to permit recovery of the platelets from any stimulation during centrifugation.

### 2.6.2. Isolation of bovine tendon collagen fibres

Bovine tendon collagen (2.5 g, Sigma) was mixed with 300 ml of 0.154 M NaCl and homogenized (Polytron model 10 OD, small probe) at 0 °C to release collagen fibres

(10 x 1 min, with a 15 min interval at room temperature in between each homogenization step). The sample was then sonicated on ice (5 x 15 s, with a 10 min interval at room temperature in between each sonication step). Collagen aggregates were removed by low-speed centrifugation (121 $g_{av.}$, 5 min) and collagen fibres were then concentrated by centrifugation (1, 100 $g_{av.}$, 20 min) and resuspended in a final volume of 5 ml of 0.154 NaCl. The concentration of collagen in the suspension (7 mg collagen/ml) was determined by drying and weighing a sample, making allowance for the amount of NaCl present.

2.6.3. Platelet aggregation experiments

Experiments were performed in a Payton aggregometer in which aggregation is measured as the decrease in attenuance (ΔD) of filtered light (609 nm filter) by the stirred platelet suspension. Platelets were stirred (1, 000 rpm) with additions for up to 3 min at 37 °C. The standard incubation mixture contained 0.90 ml of platelet suspension (3.5 to 4.5 x 10^7 platelets) and 0.1 ml of additions in 0.154 M NaCl. EHNA, lixazinone, ODQ or coformycin were dissolved in DMSO (final concentration not exceeding 0.2% (v/v)), whereas NP and DDA were dissolved in 0.154 M NaCl. After incubation for 0-1 min, platelet aggregation was initiated by adding thrombin (0.1 to 0.3 i.u./ml) or collagen (10-100 μg/ml). The course of the reaction was monitored with a Photovolt potentiometric chart recorder.

2.6.4.   Interpretation of results

Collagen · Thrombin

Incubation Period (min)
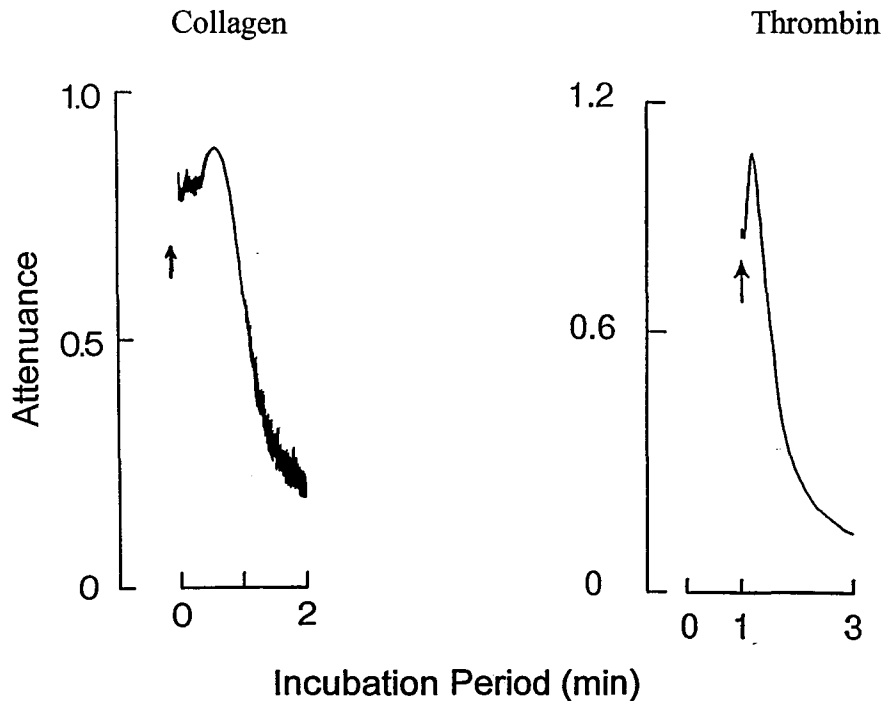
The above two traces are typical of collagen and thrombin-induced platelet aggregation. Oscillations in the tracing are caused by the swirling of disc-shaped unstimulated platelets in the stirred suspension. When platelets are stimulated (↓), a change in platelet shape from discoid to spherical results in a transient increase in attenuance of the light beam. The attenuance then rapidly decreases as the platelets aggregate. The strength of the platelet response is indicated by the relative extent of the decrease in attenuance during the aggregation phase.

3. RESULTS

3.1. Effects of EHNA on partially purified human platelet phosphodiesterases

EHNA was first recognized as a selective inhibitor of PDE2 activity when its effects were tested on PDEs isolated from human and porcine myocardium (Podzuweit *et al.*, 1995). A different array of PDEs is present in myocardial tissue (PDE1, PDE2, PDE3 and PDE4) compared to those that are present in platelets (PDE2, PDE3, and PDE5) (Hidaka and Asano, 1976; Podzuweit *et al.*, 1995), and these tissues could contain different subtypes of these isoenzyme families. Before EHNA could be utilized in the platelet studies described in this work, it was essential to establish that it is also a selective inhibitor of PDE2 when tested against the PDEs present in platelets.

To address this issue, the three known platelet PDEs were separated using a method based on the anion-exchange chromatography procedure of Hidaka and Asano (1976). As described in the Experimental section, soluble platelet proteins were chromatographed on DEAE-Sephacel resin, and fractions (0.5 ml) were eluted with a linear sodium acetate gradient (0 to 0.8 M). The PDE activities of alternate fractions were assayed with 0.5 μM [^{3}H]cAMP, 10 μM [^{3}H]cAMP or 10 μM [^{3}H]cGMP as substrates (Figure 1). The first peak of activity eluted from the column hydrolysed 10 μM [^{3}H]cGMP at a much greater rate than either concentration of [^{3}H]cAMP. The second peak of activity demonstrated robust activity with respect to either 10 μM

[^{3}H]cAMP or 10 μM [^{3}H]cGMP. The PDE activities of the first two peaks eluted were considerably greater with respect to 10 μM [^{3}H]cGMP than to 10 μM [^{3}H]cAMP. The last peak of activity that was eluted appeared to hydrolyse [^{3}H]cAMP exclusively, and its activity was by far the most prominent of the three, when the substrate was 0.5 μM [^{3}H]cAMP. Based on their respective substrate selectivities and on their order of elution from the column, the three PDEs were tentatively designated as PDE5 (peak 1), PDE2 (peak 2) and PDE3 (peak 3). Each peak of activity was pooled as indicated in Figure 1.

Further experiments were performed to establish that each peak of activity was free from contamination by other PDEs, and also to confirm that the above identifications of the pooled PDE peaks were correct. Their activities were assayed in the presence of 10 μM [^{3}H]cAMP and the following PDE inhibitors: zaprinast, an inhibitor of PDE5; EHNA, a PDE2 inhibitor; and lixazinone, which inhibits PDE3. Peak 1 (the first peak eluted from the column) was inhibited by 10 μM zaprinast to a much greater extent (85 ± 8%, mean ± S.E.M. from 4 separate experiments similar to that in Figure 2) than by either 1 μM lixazinone (41 ± 13%, mean ± S.E.M., 3 expts.) or 50 μM EHNA (18 ± 11%, mean ± S.E.M., 2 expts.). Similarly, the activities of peak 2 and peak 3 were blocked by EHNA (80 ± 1% inhibition, mean ± S.E.M., 3 expts.) and lixazinone (77 ± 4% inhibition, mean ± S.E.M., 3 expts.), respectively. Based on the responses of each enzyme pool to treatment with PDE inhibitors, it was concluded that the identities of

Figure 1: DEAE-Sephacel chromatography of human platelet PDEs

Human platelet supernatant (approximately 8 mg protein) was applied to a DEAE-Sephacel column (2 ml) and platelet proteins were eluted by a sodium acetate gradient (0-0.8 M, total volume 40 ml) in buffer containing 50 mM Tris, adjusted to pH 6.0 with acetic acid, 1 mM EDTA, 2 μM pepstatin, 40 μM chymostatin, 50 μM leupeptin, 0.5 mM PMSF, and 5 mM 2-mercaptoethanol. Samples (10 μl) of alternate eluted fractions were incubated for 5 min at 30 °C in the presence of 10 μM [^{3}H]cAMP (**a**), 0.5 μM [^{3}H]cAMP (**b**), or 10 μM [^{3}H]cGMP (**c**). The results are the amounts of [^{3}H]AMP or [^{3}H]GMP formed, expressed respectively as percentages of the [^{3}H]cAMP or [^{3}H]cGMP added. Mean values ± ranges from duplicate determinations are shown.
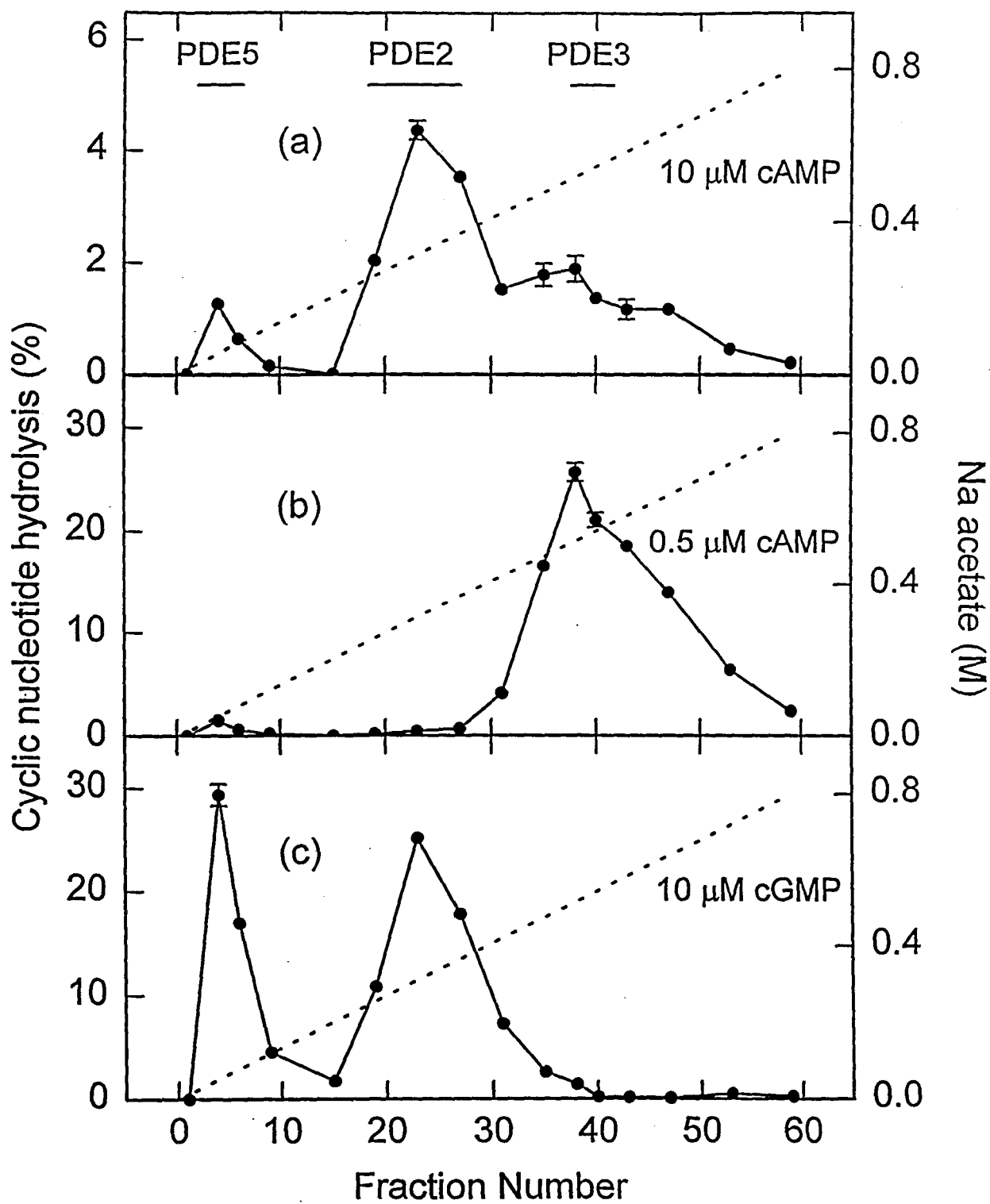
Figure 2: Selective inhibition of the eluted PDE activities

To establish the identity of each eluted PDE species (Figure 1), the effects of selective inhibitors were tested on pooled fractions. Samples (10 µl) of enzyme were incubated for 5 min at 30 °C with 10 µM [^{3}H]cAMP and the following additions: 10 µM zaprinast (an inhibitor of PDE5), 50 µM EHNA (inhibits PDE2), and 0.5 µM lixazinone (a PDE3 inhibitor). The results are the amounts of [^{3}H]AMP formed, expressed as percentages of the [^{3}H]cAMP substrate initially present. Values are means ± S.E.Q. from triplicate assays in a single experiment.
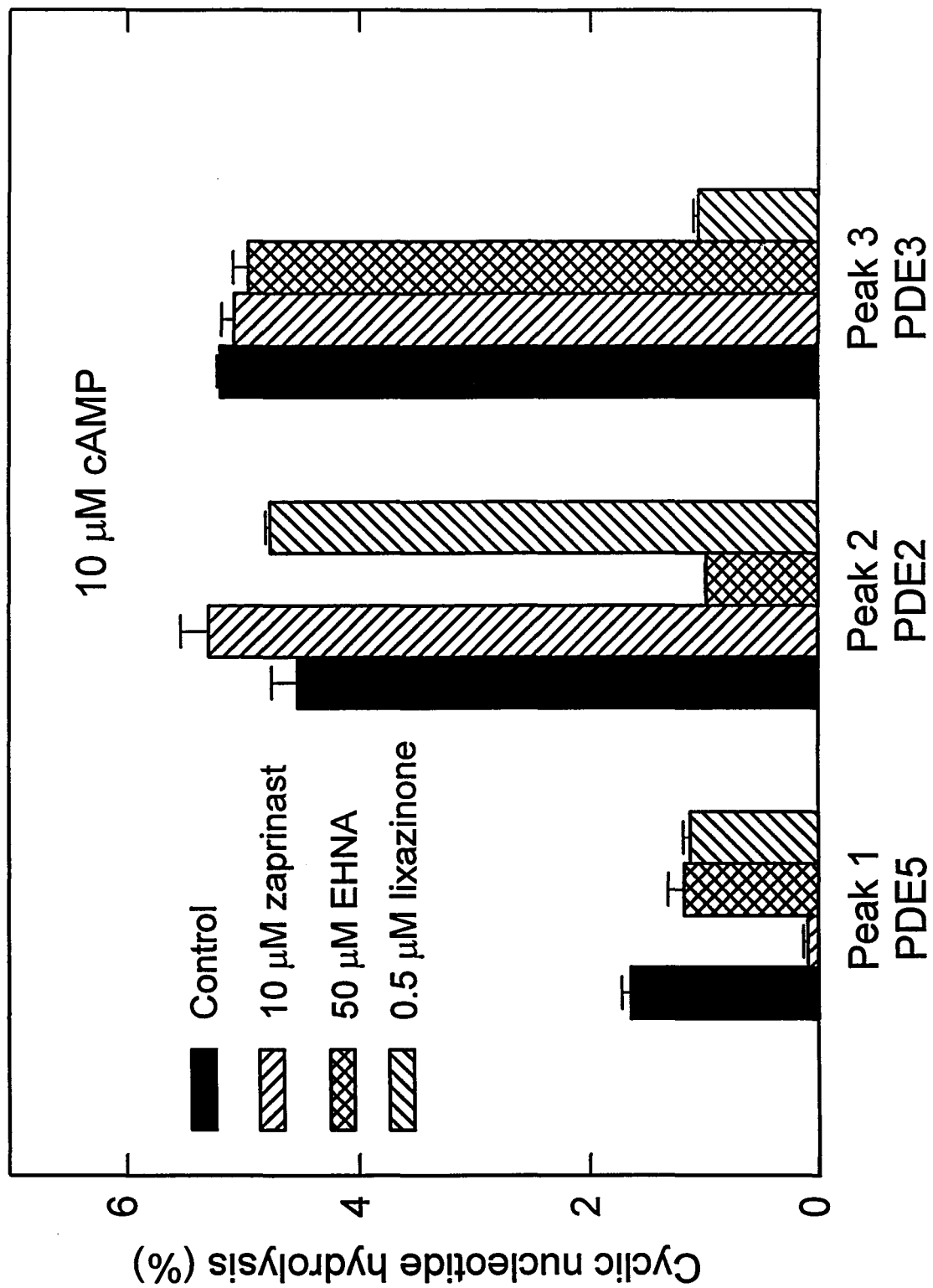
the three eluted peaks of activity were indeed PDE5, PDE2 and PDE3 (in order of elution

from the column).

Once the successful resolution of the three platelet PDEs had been verified, it

was possible to test further whether EHNA is a selective inhibitor of PDE2 in platelets.

Samples of each pooled PDE were incubated with a range of EHNA concentrations

(0.1 to 100 μM) (Figure 3). In these assays, a substrate concentration was chosen that

would result in a high level of activity for each enzyme, 0.5 μM [^{3}H]cAMP for PDE3,

and 10 μM [^{3}H]cGMP for both PDE2 and PDE5. In the cases of both PDE3 and PDE5,

no inhibitory effect of EHNA was observed at concentrations up to 30 μM, above which

very slight inhibition of both activities was observed. PDE2 was markedly inhibited by

EHNA; 50% inhibition of PDE2 activity (compared to controls without EHNA) was

observed with 1.4 μM EHNA. The results demonstrate that EHNA is a potent and

selective inhibitor of PDE2 when tested against human platelet PDEs.

PDE2 acts on both cAMP and cGMP. Several investigators have reported that

although the V_{max} of PDE2 is similar with respect to either substrate, the K_m value of

PDE2 for cAMP is approximately twice as high as that for cGMP (28 to 36 μM for

cAMP compared to 11 to 16 μM for cGMP) (Yamamoto *et al.*, 1983; Whalin *et al.*,

1988). The effectiveness of EHNA as an inhibitor of the cAMP and cGMP

phosphodiesterase activities of platelet PDE2 was therefore studied. Samples of partially

purified enzyme were incubated with a range of EHNA concentrations (0 to 100 μM)

Figure 3: Effects of EHNA on the three soluble cyclic nucleotide

phosphodiesterases isolated from human platelets

Samples (6 µl) of each partially purified PDE were incubated for 5 min at 30 °C with the

following substrates: (○) PDE2, 10 µM [^{3}H]cGMP; (□) PDE3, 0.5 µM [^{3}H]cAMP;

(▲) PDE5, 10 µM [^{3}H]cGMP, in each case in the presence of the indicated concentrations

of EHNA. Values for cyclic nucleotide hydrolysis in the presence of EHNA are

expressed as percentages of the values obtained in its absence (PDE2, $12.0 \pm 0.6\%$;

PDE3, $14.8 \pm 0.6\%$; and PDE5, $12.7 \pm 1.1\%$). Results are the means $\pm$ S.E.Q. from

triplicate assays in a single experiment.
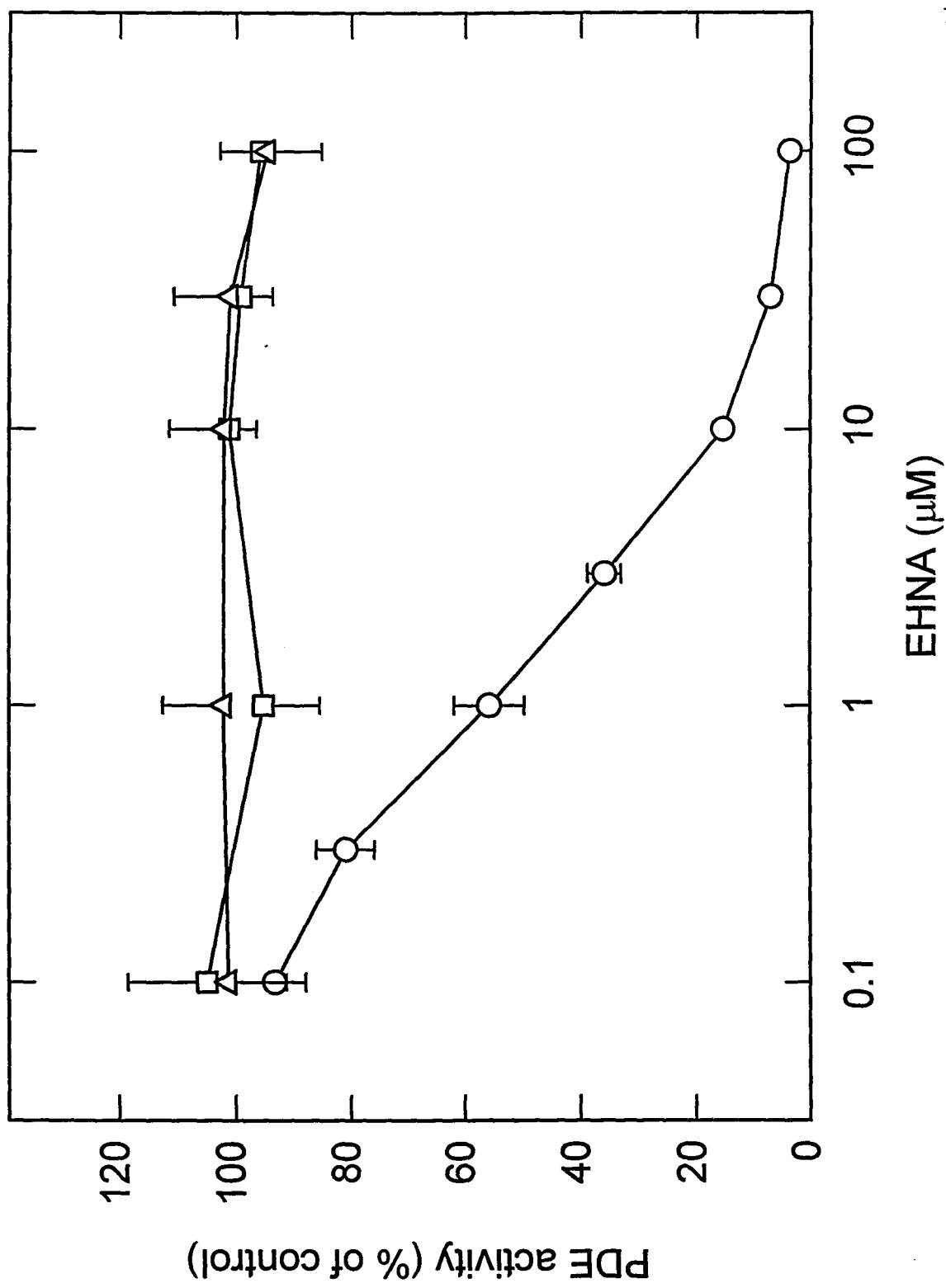
Figure 4: Effects of EHNA on the cAMP and cGMP phosphodiesterases activities of

         human platelet PDE2

Samples of partially purified PDE2 were incubated for 5 min at 30 °C in the presence of

the indicated concentrations of EHNA and either 10 μM [^{3}H]cAMP (▵) or 10 μM

[^{3}H]cGMP (○). The amounts of enzyme added were adjusted to ensure that substrate

hydrolysis was less than 15% (12 μl in the presence of [^{3}H]cAMP, 6 μl in the presence of

[^{3}H]cGMP). Values for cyclic nucleotide hydrolysis in the presence of EHNA are

expressed as percentages of the values obtained in its absence (4.4 ± 0.0% with

[^{3}H]cAMP substrate, 12.0 ± 0.6% with [^{3}H]cGMP. Results are the means ± S.E.Q. from

triplicate assays in a single experiment.
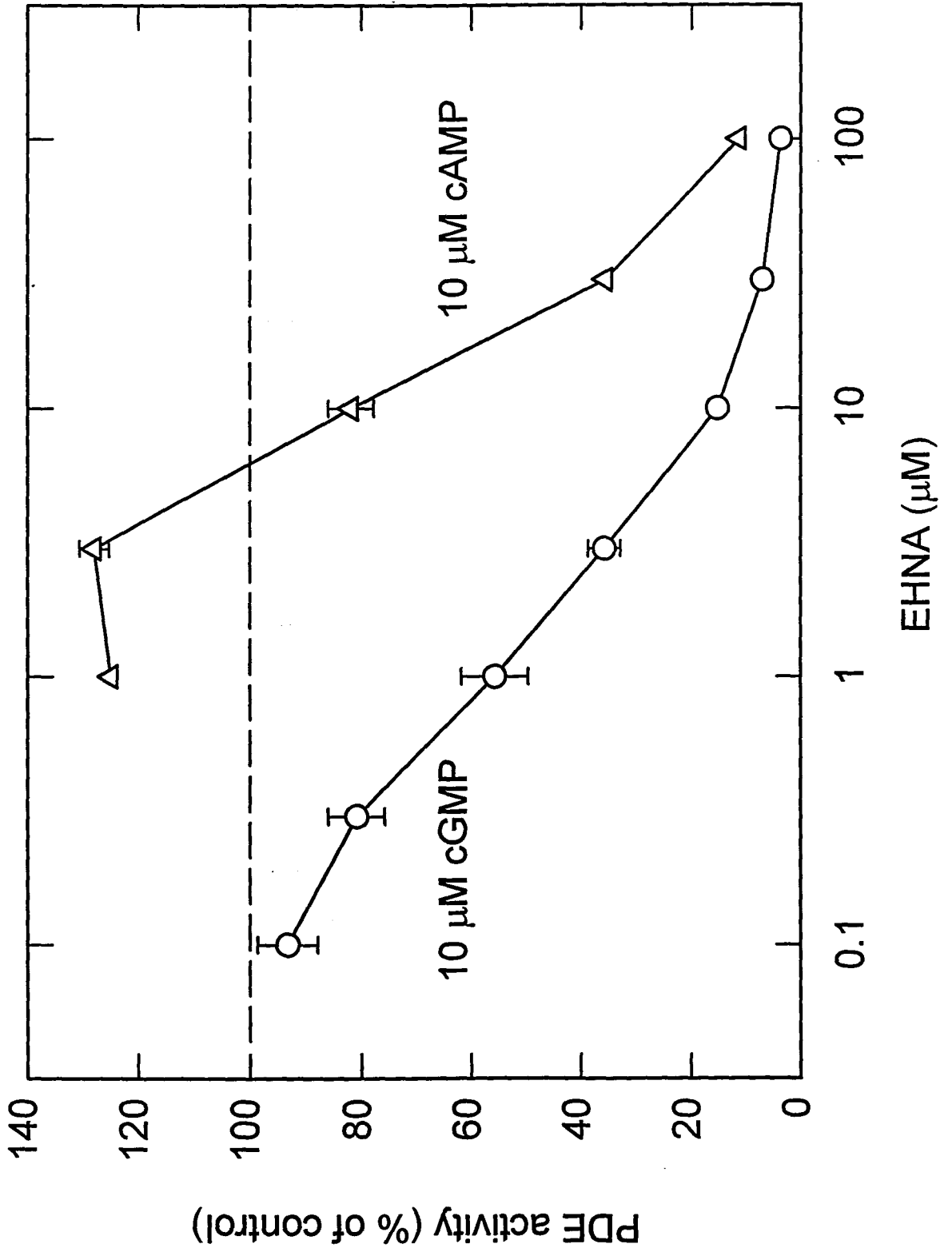
and either 10 μM [^{3}H]cAMP or 10 μM [^{3}H]cGMP, and the observed rates of substrate

hydrolysis were expressed as percentages of the rates observed in the absence of EHNA

(Figure 4). In the absence of EHNA, the rates of [^{3}H]cAMP and [^{3}H]cGMP hydrolysis

were substantially different. In this experiment, a 6 μl sample of enzyme caused 12 ± 1%

hydrolysis of 10 μM [^{3}H]cGMP, whereas a 12 μl sample of enzyme caused 4 ± 0%

hydrolysis of 10 μM [^{3}H]cAMP (mean values ± S.E.M.). In this experiment, hydrolysis

of 10 μM [^{3}H]cAMP was weakly but clearly potentiated by low concentrations of EHNA

(Figure 4). This effect was quite variable in magnitude, and over all the experiments

performed was not statistically significant (11.5 ± 8.4 % increase with 1 μM EHNA and

13.2 ± 9.2 % increase with 3 μM EHNA (means ± S.E.M., three separate experiments).

As the concentration of EHNA was increased, inhibition (compared to controls without

EHNA) was observed and reached 50% with 22 μM compound. When the substrate was

10 μM [^{3}H]cGMP, no stimulatory effect of EHNA was noted and the IC_{50} value for

inhibition of cGMP hydrolysis by EHNA was 1.4 μM, much lower than observed with

[^{3}H]cAMP. The results suggest that EHNA is a more effective inhibitor of the cGMP

than of the cAMP phosphodiesterase activity of PDE2, when equivalent low substrate

concentrations are used.

The hallmark of PDE2 activity is potent stimulation of the hydrolysis of

cAMP by low concentrations of cGMP (e.g. 0.1 to 3 μM cGMP) (Sheth and Colman,

1995a; Manganiello *et al.*, 1990). The effects of cGMP (1 μM) on the inhibition of the

Figure 5: Effects of cGMP on the inhibition by EHNA of the cAMP

phosphodiesterase activity of human platelet PDE2

Samples of partially purified PDE2 (12 μl) were incubated for 5 min at 30 °C with 10 μM [^{3}H]cAMP in the absence (O) or presence (●) of 1 μM cGMP, in each case in the presence of the indicated concentrations of EHNA. Values for [^{3}H]cAMP hydrolysis are expressed as percentages of the [^{3}H]cAMP initially present and are means ± S.E.M. of three determinations in a single experiment.

cAMP hydrolysis (%)

EHNA (μM)
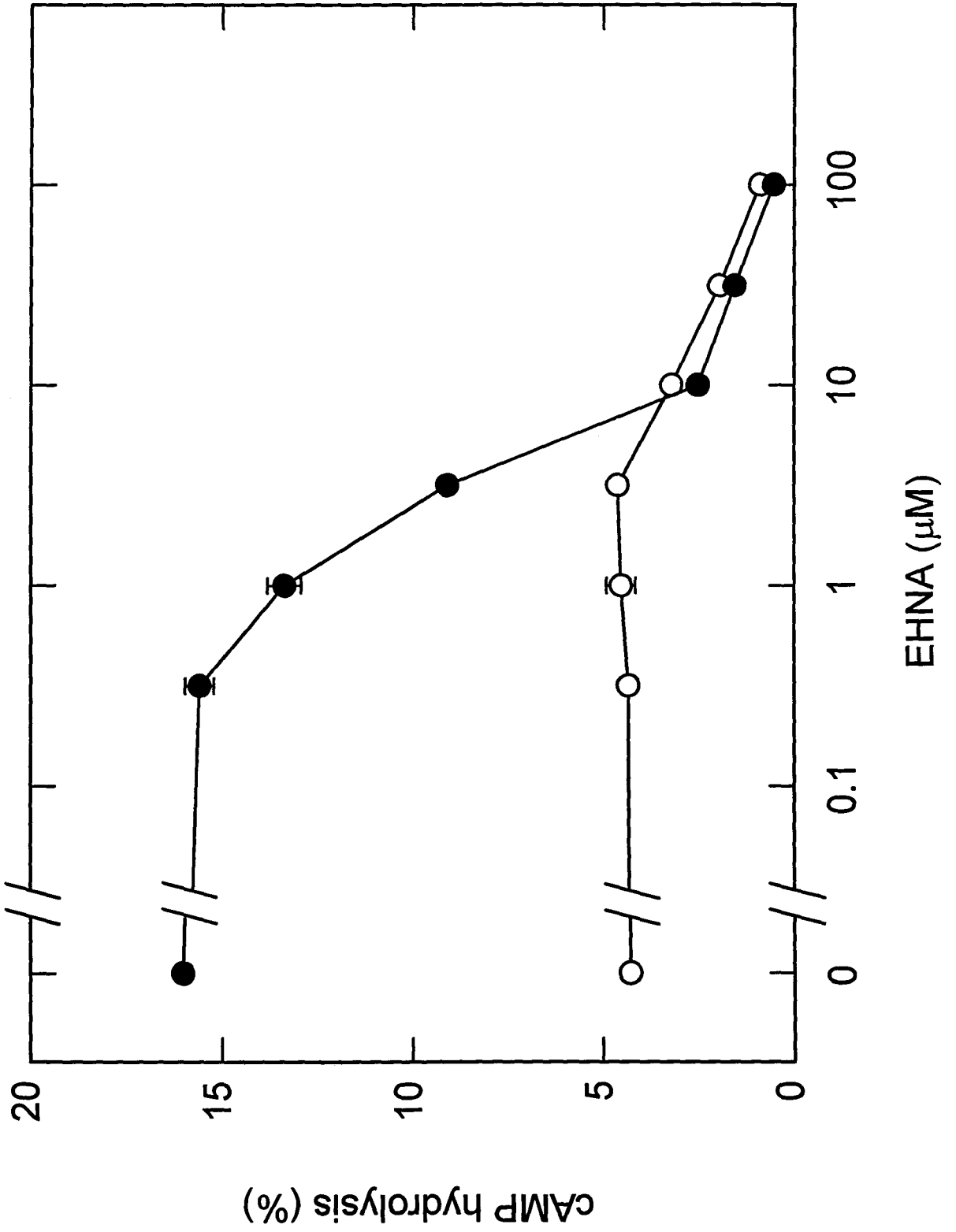
[^{3}H]cAMP phosphodiesterase activity of PDE2 by EHNA were therefore tested (Figure 5). In the absence of EHNA, addition of 1 μM cGMP increased the [^{3}H]cAMP phosphodiesterase activity of PDE2 against 10 μM [^{3}H]cAMP by almost fourfold (380 ± 10%, mean ± range, 2 expts.). Inhibition of [^{3}H]cAMP hydrolysis was observed at lower concentrations of EHNA in the presence of cGMP (IC$_{50}$, 3.3 μM) than in its absence (IC$_{50}$, 15 μM EHNA); i.e. EHNA is a more effective inhibitor of the [^{3}H]cAMP phosphodiesterase activity of PDE2 in the presence of cGMP. At concentrations of EHNA greater than 10 μM, [^{3}H]cAMP was hydrolysed at similar absolute rates, whether cGMP was present or not (Figure 5).

The converse experiments were also conducted, in which the effects of EHNA on the stimulation by cGMP of the cAMP phosphodiesterase activity of PDE2 were studied. These investigations were initially performed with a relatively high concentration of EHNA (30 μM), well above the IC$_{50}$ values for EHNA that had been measured with either substrate. Samples of partially purified PDE2 were incubated with 10 μM [^{3}H]cAMP in the absence or presence of 30 μM EHNA, and PDE2 activity was stimulated by 0.2 or 2 μM cGMP (Figure 6). EHNA partially inhibited PDE2 activity in the absence of cGMP, but as the concentration of cGMP was increased the stimulation of phosphodiesterase activity by the latter and the inhibition exerted by EHNA both increased markedly. Thus EHNA appeared to block the stimulatory effect of cGMP, suggesting that EHNA could have effects at the regulatory cGMP-binding site of PDE2.

Figure 6: Effect of EHNA on the stimulation by cGMP of the cAMP

   phosphodiesterase activity of PDE2

Samples of partially purified enzyme (12 μl) were incubated for 5 min at 30 °C with

10 μM [³H]cAMP in the absence (solid bars) or presence (hatched bars) of 30 μM

EHNA, in each case in the presence of the indicated concentrations of cGMP. Values for

[³H]cAMP hydrolysis are expressed as percentages of the [³H]cAMP initially present and

are means ± S.E.M. of three determinations in a single experiment.
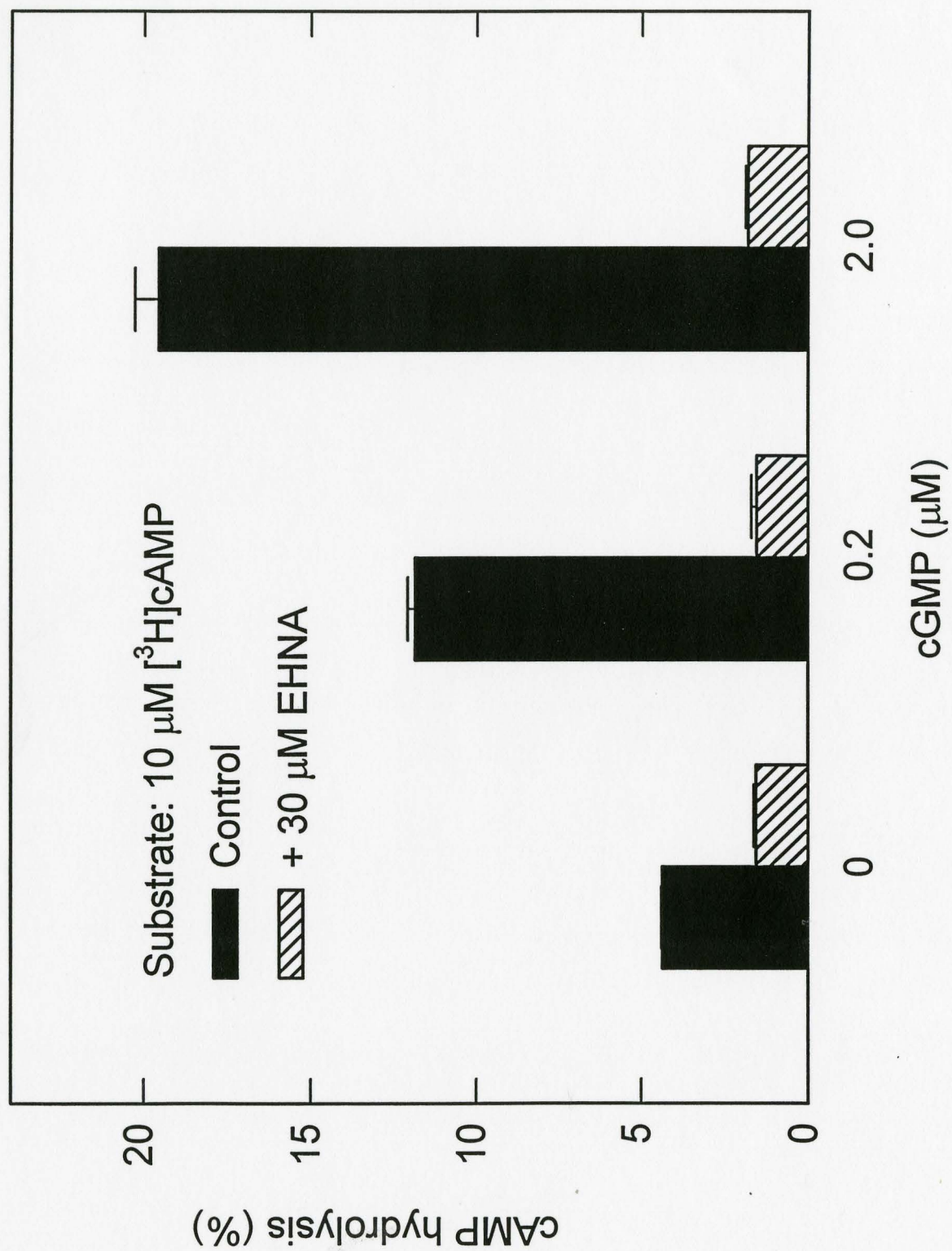
To explore this possibility in greater detail, a related experiment was performed in which the effects of a low concentration of EHNA on the stimulation of the cAMP phosphodiesterase activity of PDE2 by cGMP were studied. Samples of partially purified enzyme were incubated with 10 μM [^{3}H]cAMP and a range of cGMP concentrations (0 to 100 μM), in each case in the absence or presence of 2 μM EHNA (Figure 7). This concentration of EHNA is well below the IC_{50} value of EHNA observed in the presence of 10 μM [^{3}H]cAMP alone (IC_{50}, 18 ± 4 μM, mean ± range, 2 expts.), and was chosen in an attempt to isolate any potential effect of EHNA on the regulatory site of PDE2. In the absence of EHNA, [^{3}H]cAMP hydrolysis was increased more than threefold by cGMP; maximal stimulation of [^{3}H]cAMP hydrolysis was observed in the presence of 3 μM cGMP. When the cGMP concentration was increased above 10 μM, a decrease in the stimulatory effect was observed, presumably as a result of cGMP competing with [^{3}H]cAMP at the active site of PDE2.

As shown in Figure 8, in which the results in Figure 7 are replotted to show the inhibitory effects of EHNA on stimulation by different cGMP concentrations, the effect of EHNA was largely independent of the concentration of cGMP. This suggests that EHNA is not acting at the cGMP-binding regulatory site but rather that cGMP may modify the inhibitory action of EHNA at the active site. Further analysis will require detailed kinetic studies with more highly purified enzyme.

Figure 7: Effects of a low concentration of EHNA on the cGMP-stimulated cAMP

phosphodiesterase activity of PDE2

Samples of partially purified enzyme (12 μl) were incubated for 5 min at 30 °C with

10 μM [^{3}H]cAMP in the absence (△) or presence (▲) of 2 μM EHNA, in each case in the

presence of the indicated concentrations of cGMP. Values are [^{3}H]cAMP hydrolysis

expressed as percentages of the [^{3}H]cAMP initially present and are means ± S.E.M. of

three determinations in a single experiment.

cGMP (µM)

cAMP hydrolysis (%)
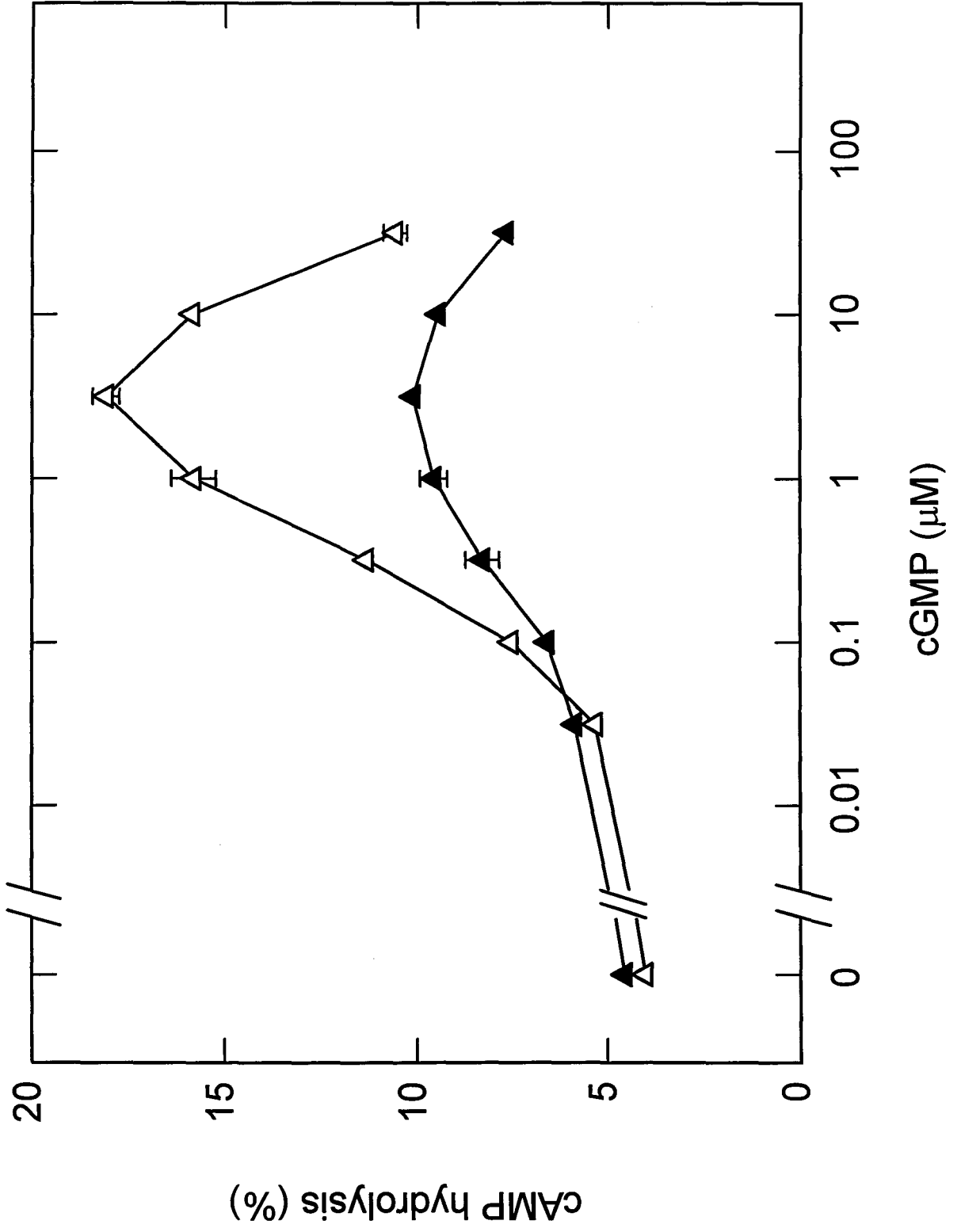
Figure 8: **Effects of cGMP concentration on the inhibition of the cAMP**

phosphodiesterase activity of PDE2 by a low concentration of EHNA

The values from Figure 7 were replotted to express the inhibition of the stimulatory effect of cGMP exerted by 2 μM EHNA. [³H]cAMP hydrolysis observed in the absence of EHNA and cGMP was substracted from all values. Results are means ± S.E.Q from triplicate measurements in a single experiment.
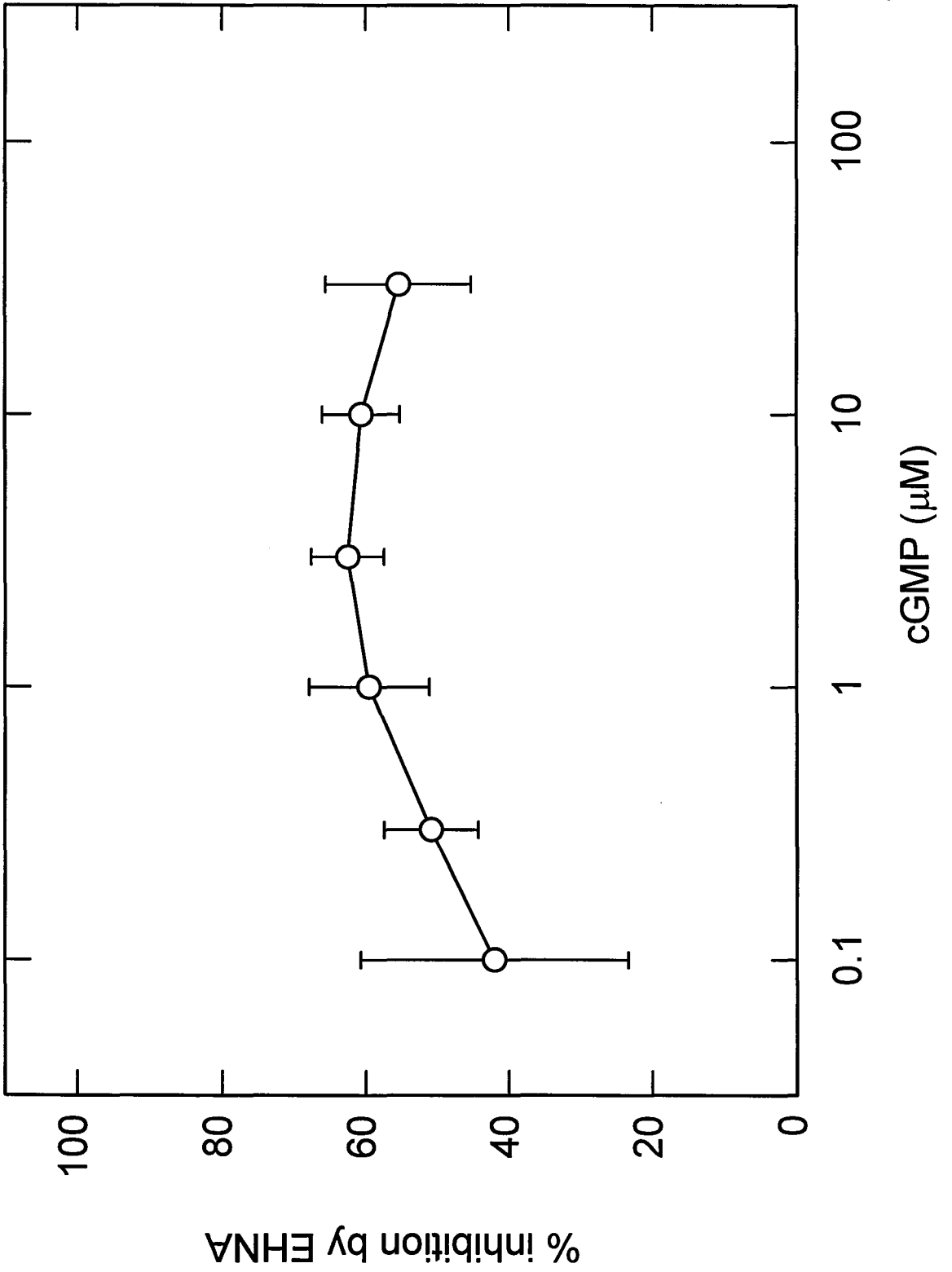
3.2.   The role of PDE2 in regulating NP-induced changes in platelet cyclic nucleotide levels

The two platelet cAMP phosphodiesterases respond to cGMP in opposite ways. Thus, PDE2 is stimulated by cGMP, causing a decrease in cAMP levels; whereas PDE3 is inhibited by cGMP, thereby increasing cAMP concentrations. Earlier experiments in this laboratory investigated the effects that cGMP has on cAMP accumulation in prelabelled human platelets (Section 2.5). In these studies, NP (an activator of guanylyl cyclase) exerted biphasic effects on cAMP accumulation; lower concentrations of NP (0.1 to 1 μM) increased [^{3}H]cAMP levels, but this effect was attenuated at higher concentrations of NP (10 to 100 μM) (M. M. L. Davidson and R. J. Haslam, unpublished). The cause of the stimulatory component of the effects of NP on platelet cAMP levels was identified as inhibition of PDE3 by cGMP (Maurice and Haslam, 1990a), but the mechanism underlying the inhibitory phase of the biphasic effect of NP had not yet been determined. Since cGMP activates PDE2, the involvement of PDE2 in the inhibitory phase seemed plausible, and was explored in this study using EHNA (Figure 9). Prelabelled human platelets were incubated with a range of NP concentrations (0 to 100 μM) in the presence and absence of EHNA (20 μM), and the resulting changes in platelet [^{3}H]cAMP and [^{3}H]cGMP levels were measured. In the absence of EHNA, a biphasic effect of NP on [^{3}H]cAMP accumulation was observed, consistent with earlier studies, and the maximum stimulatory effect was exerted by

Figure 9: Effects of different NP concentrations on the accumulation of cyclic

nucleotides in human platelets in the presence or absence of lixazinone

and EHNA

Platelet metabolic nucleotide pools were prelabelled by incubation with [^{3}H]adenine and

[^{3}H]guanine. Samples of labelled platelet suspension were incubated for 1 min at 37 °C

with the indicated concentrations of NP and either no further additions (○), 20 μM EHNA

(to inhibit PDE2) (●), 1 μM lixazinone (to inhibit PDE3) (▽), or 20 μM EHNA + 1 μM

lixazinone (▼). Platelet [^{3}H]cAMP and [^{3}H]cGMP were isolated and separated as

described in the Experimental section (2.5.3.). Total platelet [^{3}H]ATP and [^{3}H]GTP were

measured according to the cellulose thin layer chromatographic method described in the

Experimental section (2.5.4.). The results for platelet [^{3}H]cAMP (**a**) and [^{3}H]cGMP (**b**)

are expressed as percentages of total platelet [^{3}H]ATP (1.6 x 10^6 dpm/sample) and

[^{3}H]GTP (1.5 x 10^6 dpm/sample), respectively. Experimental values are the means ±

S.E.M. of triplicate measurements in a single experiment.
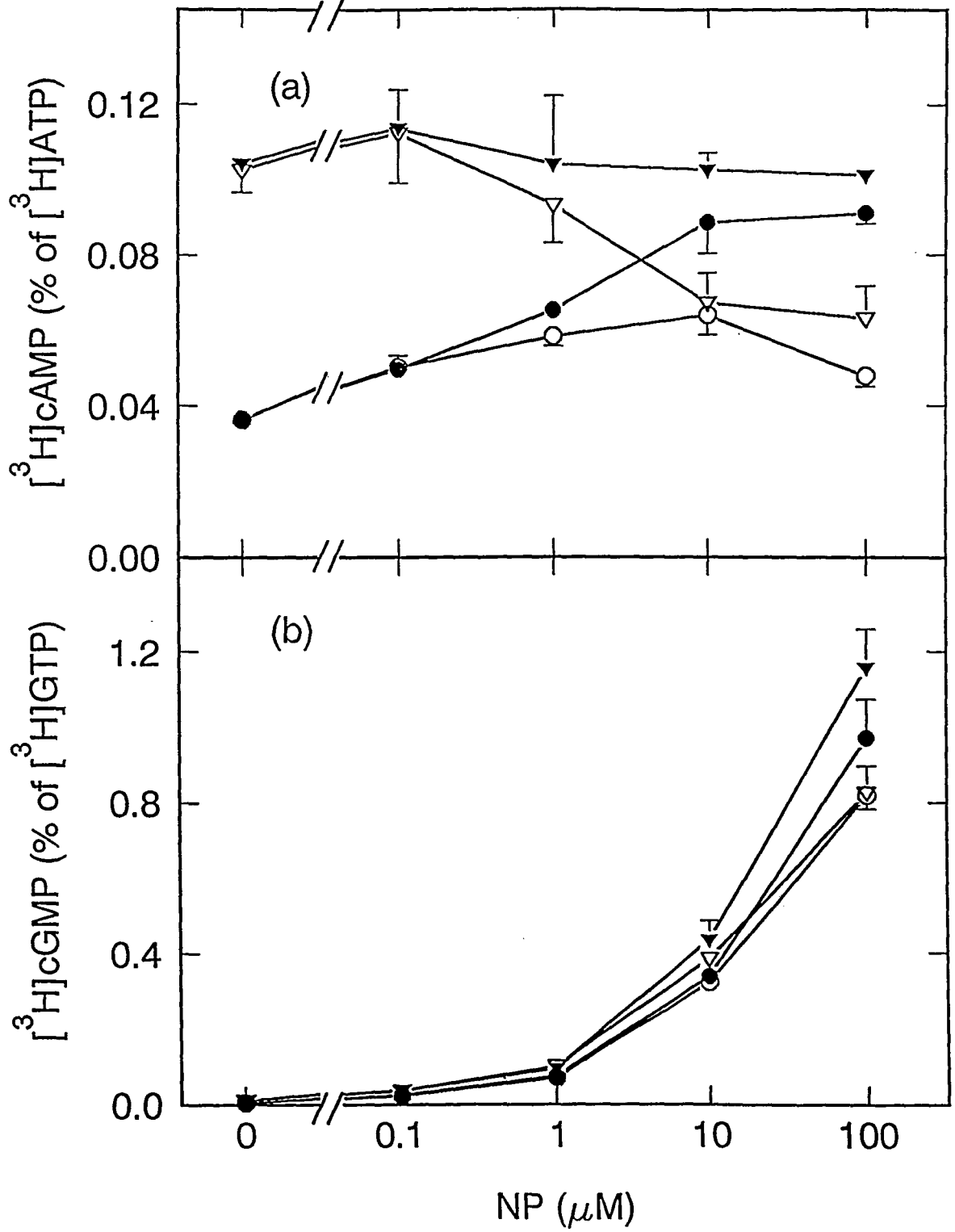
10 μM NP. In all similar experiments performed in this laboratory (the majority were of

carried out by E. K. Jang), the following increases in [^{3}H]cAMP have been observed

(means ± S.E.M.): 29 ± 5% with 0.1 μM NP (3 expts.), 47 ± 5% with 1 μM NP (8

expts.), 49 ± 6% with 10 μM NP (9 expts.) and 32 ± 6% with 100 μM NP (13 expts.). In

those experiments in which the last two NP concentrations were studied, a statistically

significant reduction in [^{3}H]cAMP accumulation was observed when the NP

concentration was increased from 10 to 100 μM ($P < 0.05$, two-sided paired t test, $n = 8$).

As expected, increasing the NP concentration resulted in pronounced stimulation of

platelet [^{3}H]cGMP accumulation, reaching 1.3 ± 0.2% of the total [^{3}H]GTP at 100 μM

NP (mean ± S.E.Q., 15 expts.). To determine if PDE2 mediated the reduction in

[^{3}H]cAMP accumulation observed when the NP concentration was increased from 10 to

100 μM, prelabelled platelets were incubated with a range of NP concentrations in the

presence of 20 μM EHNA (Figure 9). EHNA had no effect on [^{3}H]cAMP accumulation

when the NP concentration was 0.1 μM or lower, but as the concentration of NP was

increased, the accumulation of [^{3}H]cAMP was enhanced and reached a maximum with 10

to 100 μM NP. At the latter concentration, EHNA increased [^{3}H]cAMP levels by 125 ±

14% (compared to 100 μM NP alone; mean ± S.E.M., 11 expts.). This increase was

statistically significant ($P < 0.001$, two-sided paired t test, 11 expts.). The results show

that at higher NP concentrations, activation of PDE2 restricted platelet [^{3}H]cAMP

accumulation, and therefore demonstrate that PDE2 mediates the inhibitory component of

Figure 10: **Effects of EHNA on the accumulation of cyclic nucleotides in human platelets caused by NP and lixazinone**

Platelet metabolic nucleotide pools were prelabelled by incubation with [^{3}H]adenine and [^{3}H]guanine. Samples of labelled platelet suspension were incubated for 1 min at 37 °C with the indicated concentrations of EHNA (to inhibit PDE2) and either no further additions (○), 100 μM NP (●), 1 μM lixazinone (to inhibit PDE3) (△), or 100 μM NP + 1 μM lixazinone (▲). Platelet [^{3}H]cAMP (**a**) and [^{3}H]cGMP (**b**) were assayed and expressed as percentages of total [^{3}H]ATP (1.4 x 10^6 dpm/sample) and total [^{3}H]GTP (1.9 x 10^6 dpm/sample), respectively. Values are the means ± S.E.M. of triplicate measurements in a single experiment.
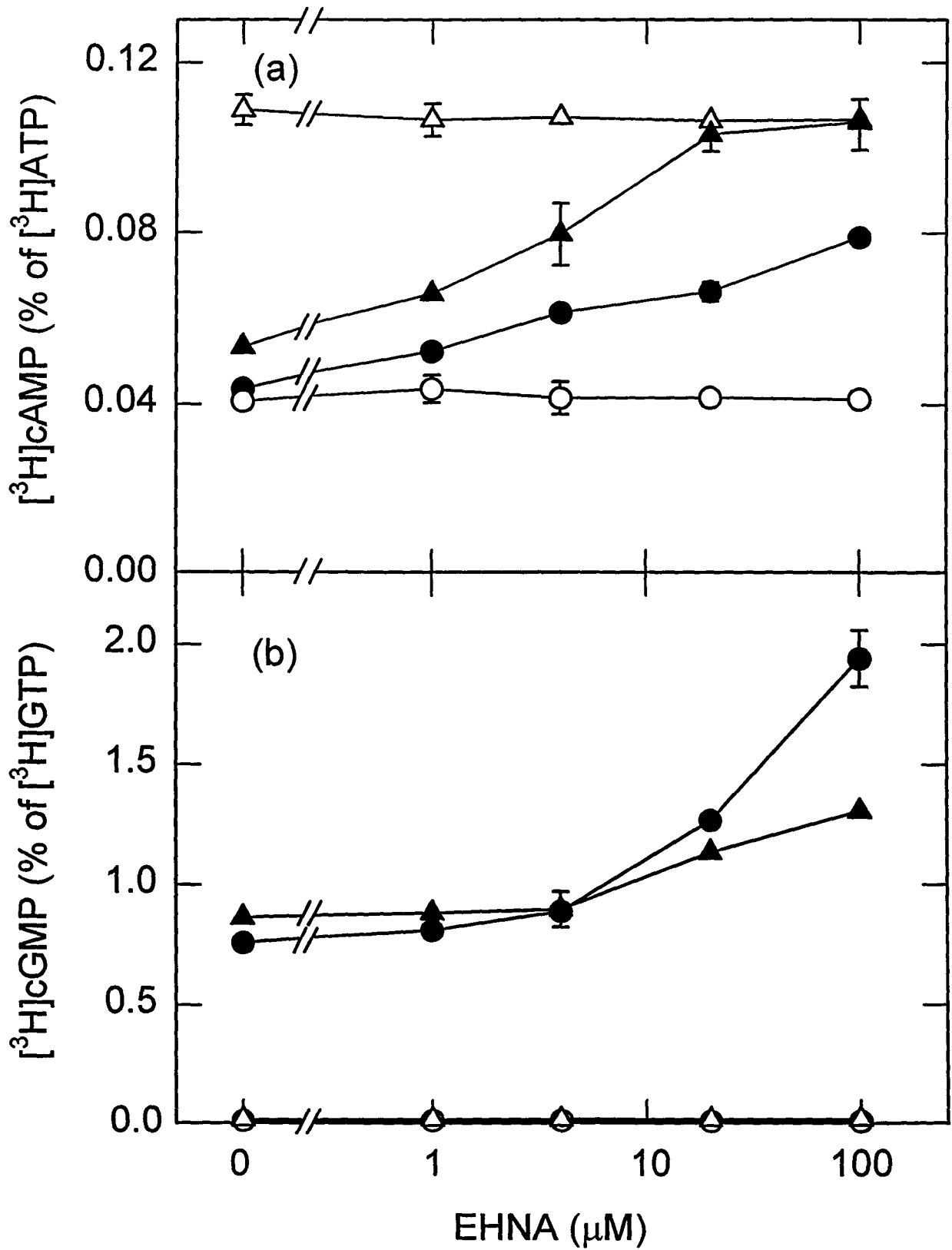
the biphasic effect of NP.

To examine the effects of a range of EHNA concentrations on [^{3}H]cAMP accumulation when PDE2 was strongly stimulated by cGMP, prelabelled platelets were incubated with 100 μM NP and EHNA (0 to 100 μM) (Figure 10). EHNA alone caused no effect on [^{3}H]cAMP or [^{3}H]cGMP accumulation over the range of concentrations examined. In the presence of NP, the accumulation of [^{3}H]cGMP was increased by EHNA concentrations of 30 μM and greater; a maximum increase of 113 ± 28% increase above that with 100 μM NP alone (mean ± S.E.M., 12 expts.) was observed with 100 μM EHNA. This indicates that in these conditions PDE2 plays a significant role in cGMP hydrolysis. In the absence of EHNA, 100 μM NP caused no significant increase in [^{3}H]cAMP accumulation in this experiment (an unusual result). However, as the concentration of EHNA was increased from 1 to 100 μM, the accumulation of [^{3}H]cAMP in the presence of NP was progressively enhanced, demonstrating that the inhibitory effect of NP on [^{3}H]cAMP accumulation was abolished by inhibiting PDE2.

As mentioned above, cGMP is both an activator of PDE2 and an inhibitor of PDE3. To dissociate the effects of NP (or cGMP) on PDE3 from its effects on PDE2, prelabelled platelets were also incubated with a range of NP concentrations in the presence of lixazinone (1 μM) to inhibit PDE3 (Figure 9). In the absence of NP, lixazinone did not increase [^{3}H]cGMP, but inhibition of PDE3 resulted in a large increase in [^{3}H]cAMP (177 ± 15%, mean ± S.E.M., 4 expts.). As the concentration of NP was

increased above 0.1 μM, the stimulatory effect of lixazinone was attenuated and reached

a minimum with 100 μM NP, at which concentration platelet [^{3}H]cAMP levels had fallen

to 55 $\pm$ 5% of those observed with lixazinone alone (mean $\pm$ S.E.M., 4 expts.). When

prelabelled platelets were incubated with EHNA (20 μM) in addition to lixazinone, the

inhibitory effect of NP on lixazinone-induced [^{3}H]cAMP accumulation was completely

blocked (Figure 9), demonstrating that activation of PDE2 by NP (or cGMP) was

responsible for the inhibition of [^{3}H]cAMP accumulation.

To verify that activation of PDE2 opposes the increases in platelet [^{3}H]cAMP

caused by inhibition of PDE3, the effects of a range of EHNA concentrations were tested

on prelabelled platelets incubated with lixazinone in the presence and absence of 100 μM

NP (Figure 10). Inhibition of PDE3 by lixazinone caused a large increase in [^{3}H]cAMP

accumulation similar to that observed in other experiments. As the concentration of

EHNA was increased, the inhibitory effect of NP was diminished until at 20 μM EHNA,

an effect of NP was no longer observed, signifying that the inhibitory effect of NP on

[^{3}H]cAMP accumulation was attributable to the activation of PDE2.

These experiments demonstrated that addition of NP activates PDE2 in intact

human platelets. This restricts the increases in [^{3}H]cAMP and to a lesser extent

[^{3}H]cGMP that are induced by NP. Moreover, activation of PDE2 by NP greatly reduces

the effects of inhibition of PDE3 on cAMP accumulation. The changes in platelet

[^{3}H]cAMP and [^{3}H]cGMP observed when PDE2 was inhibited by EHNA illustrate that

Figure 11:   Interactions between NP, lixazinone and EHNA in their effects on

thrombin-induced platelet aggregation

Human platelets were isolated and washed as described in the Experimental section. Samples of platelet suspension were stirred for 1 min at 37 °C in the absence (**a-d**) or presence (**e-h**) of 20 μM EHNA and the following other additions: (**a, e**), none; (**b, f**), 10 μM NP; (**c, g**), 1 μM lixazinone; (**d, h**), 10 μM NP + 1 μM lixazinone. Platelet aggregation was then initiated by the addition of human thrombin (final concentration 0.3 i.u./ml) (arrow) and recorded with an aggregometer. Tracings of the recordings are shown.
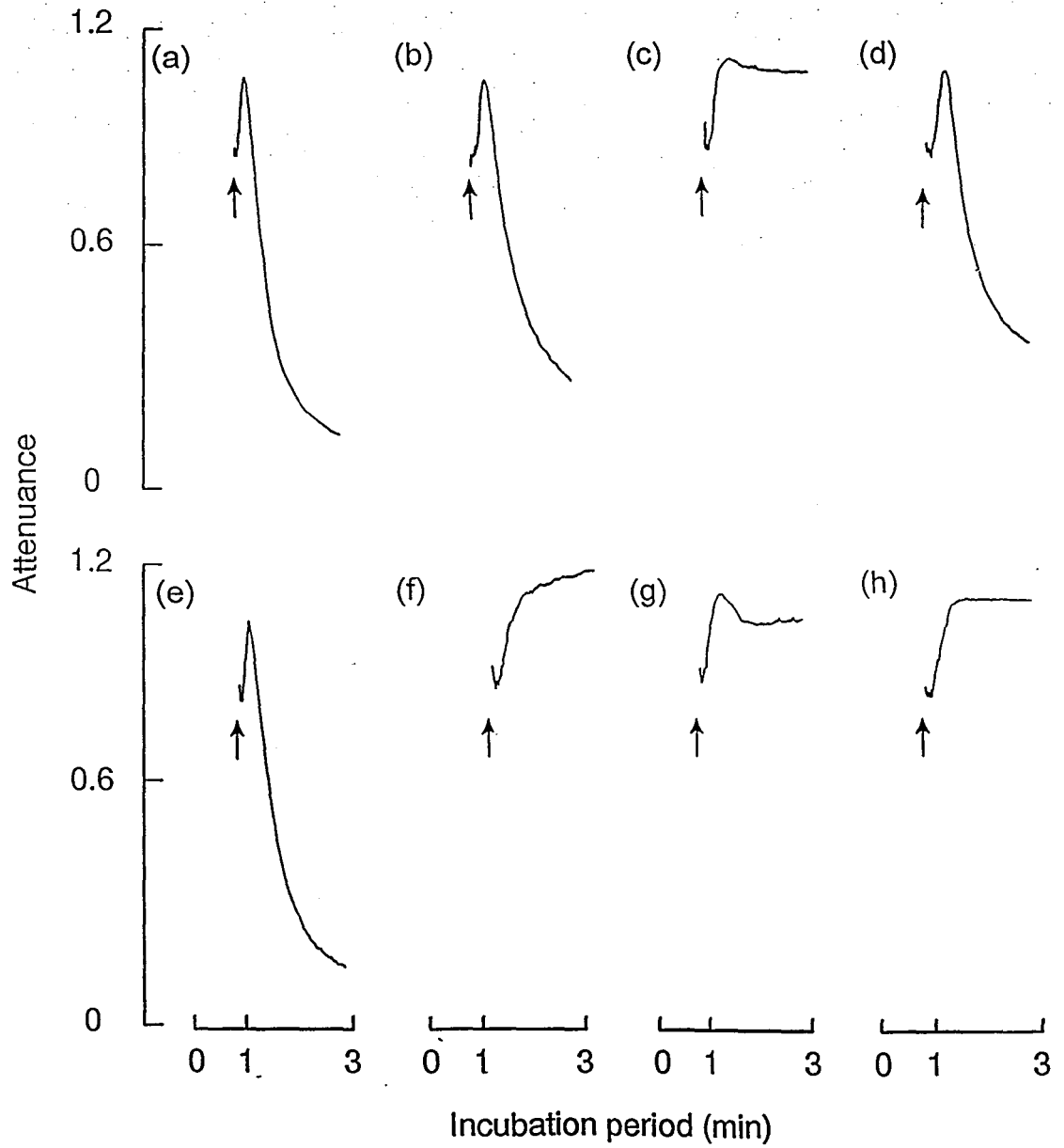
this enzyme plays a major role in the effects of NP on platelet cyclic nucleotide

metabolism.

3.3.   Interactions between PDE2 and PDE3 in thrombin-induced platelet

aggregation

Cyclic nucleotides are crucial regulators of platelet function. Platelet

responses, such as aggregation, are inhibited by increases in cAMP and cGMP levels

(Haslam *et al.*, 1978a, 1980). Because the above experiments demonstrated an important

role for PDE2 in attenuating the increases in platelet cyclic nucleotides caused by NP

(Figures 8 and 9), the role of this enzyme in the effects of NP on thrombin-induced

platelet aggregation was investigated (Figure 11). After incubating platelets for 1 min at

37 °C with NP and other additions, aggregation was stimulated by thrombin. The

concentration of thrombin was adjusted so that 10 µM NP caused only partial inhibition

of aggregation (Figure 11, **a** and **b**). Although EHNA alone exerted no effect on

aggregation, it increased the inhibitory effect of NP to the point that platelet aggregation

was completely blocked (Figure 11, **e** and **f**). Thus, PDE2 activity reduced the inhibitory

potential of NP.

PDE3 is the major cAMP phosphodiesterase in resting platelets (Sheth and

Colman, 1995a), and, as expected, the increases in cAMP caused by inhibiting this

enzyme (Figures 8 and 9) markedly inhibited platelet aggregation (Figure 11 **c**). This

effect was overcome by addition of NP (Figure 11 **d**). The inhibition of the effects of

lixazinone by NP was blocked by EHNA, indicating that it was mediated by activation of

PDE2 (Figure 11 **h**).

In experiments with partially purified enzyme, a variable and small

stimulation of the cAMP phosphodiesterase activity of PDE2 by low EHNA

concentrations was observed (Figure 4). A weak stimulatory effect of EHNA on platelet

aggregation was also observed when thrombin-induced platelet aggregation was

prevented by 1 μM lixazinone (Figures 11 and 12). Inhibition by lixazinone was slightly

reduced by 20 μM EHNA (Figure 11, **c** and **g**) but 5 μM EHNA was more effective

(Figure 12, **c** and **d**). The apparent stimulatory effect of EHNA on cAMP hydrolysis by

PDE2 may be correlated with this reduction in the inhibitory effect of lixazinone on

thrombin-induced platelet aggregation.

The inhibitory effect of NP on platelet aggregation was markedly increased by

EHNA (Figure 11, **b** and **f**). Platelet aggregation can be inhibited by increases in either

cAMP or cGMP, and the NP-induced accumulation of both cyclic nucleotides (but

particularly cAMP) is increased in the presence of EHNA (Figures 9 and 10). To address

the question of whether it is cAMP or cGMP that is responsible for the potentiated

inhibitory effect observed in the presence of EHNA, an inhibitor of adenylyl cyclase,

DDA, was utilized. In the presence of DDA, increases in cAMP should be diminished

and cAMP-dependent reactions impaired. Accordingly, the effects of DDA (100 μM) on

Figure 12:   **Stimulation of thrombin-induced platelet aggregation by a low EHNA**

concentration in the presence of lixazinone

Human platelets were isolated and washed as described in the Experimental section.

Samples of platelet suspension were incubated for 1 min at 37 °C with no further

additions (**a**) or in the presence of 20 µM EHNA (**b**), 1 µM lixazinone (**c**), or EHNA +

lixazinone. Platelet aggregation was then initiated by the addition of human thrombin

(final concentration 0.3 i.u./ml) (arrow) and recorded with an aggregometer. Tracings of

the recordings are shown.
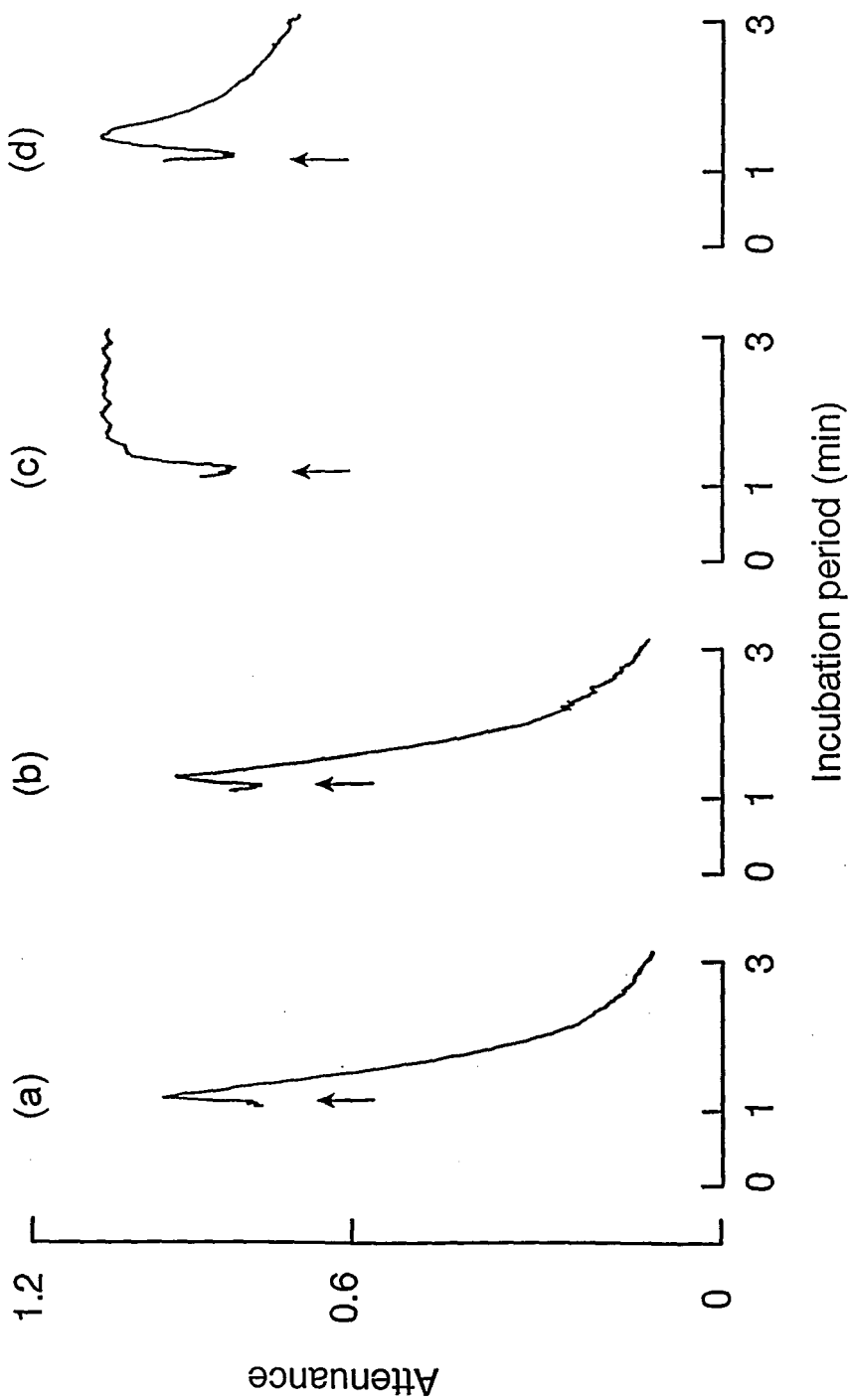
thrombin-induced platelet aggregation in the presence of NP (10 μM) and EHNA

(20 μM) were examined (Figure 13). Aggregation induced by thrombin alone was

unaffected by DDA (Figure 13, **a** and **b**). When inhibition of aggregation by NP was

adjusted to be partial, DDA had only a minor effect in reducing this inhibition (Figure 13,

c and **d**). As had been noted before (Figure 11), EHNA greatly increased this inhibitory

effect of NP (Figure 13 **e**), while exerting no effect by itself (data not shown). The

synergistic inhibition of aggregation caused by addition of NP with EHNA was

diminished by DDA (Figure 13 **f**), showing that this was mediated at least in part by

cAMP.

To investigate the effects of the corresponding conditions on platelet cyclic

nucleotides, prelabelled platelets were incubated for 1 min with 10 μM NP, 20 μM

EHNA, or NP and EHNA in each case in the absence or presence of 100 μM DDA (Table

2). Significant increases in platelet [^{3}H]cGMP levels were only observed in the presence

of NP. As observed before, NP-induced accumulation of [^{3}H]cGMP was slightly

increased by EHNA (11 ± 5% above that with NP alone, mean ± S.E.M., 6 expts.).

Unexpectedly, platelet [^{3}H]cGMP levels were also slightly increased by DDA in the

presence of NP (9 ± 1%, $P < 0.05$, 2-sided paired t-test, 3 expts.) or of NP and EHNA (10

± 4%; mean ± S.E.M., 3 expts.). The effects of 10 μM NP on platelet [^{3}H]cAMP in the

presence and absence of EHNA agreed with earlier observations. NP alone increased

[^{3}H]cAMP accumulation (54 ± 7%, mean ± S.E.M., 3 expts.). This

Figure 13: **Effect of DDA on the inhibition of thrombin-induced platelet aggregation by NP and EHNA**

Human platelets were isolated and washed as described in the Experimental section. Samples of platelet suspension were incubated for 1 min at 37 °C in the absence (**a, c, e**) or presence (**b, d, f**) of 100 μM DDA and the following other additions: none (**a, b**); 10 μM NP (**c, d**); 10 μM NP + 20 μM EHNA (**e, f**). Platelet aggregation was then initiated by the addition of human thrombin (final concentration 0.125 i.u./ml) (arrow) and recorded with an aggregometer. Tracings of the recordings are presented. The addition of 20 μM EHNA or 20 μM EHNA + 100 μM DDA had no effect on platelet aggregation (results not shown).
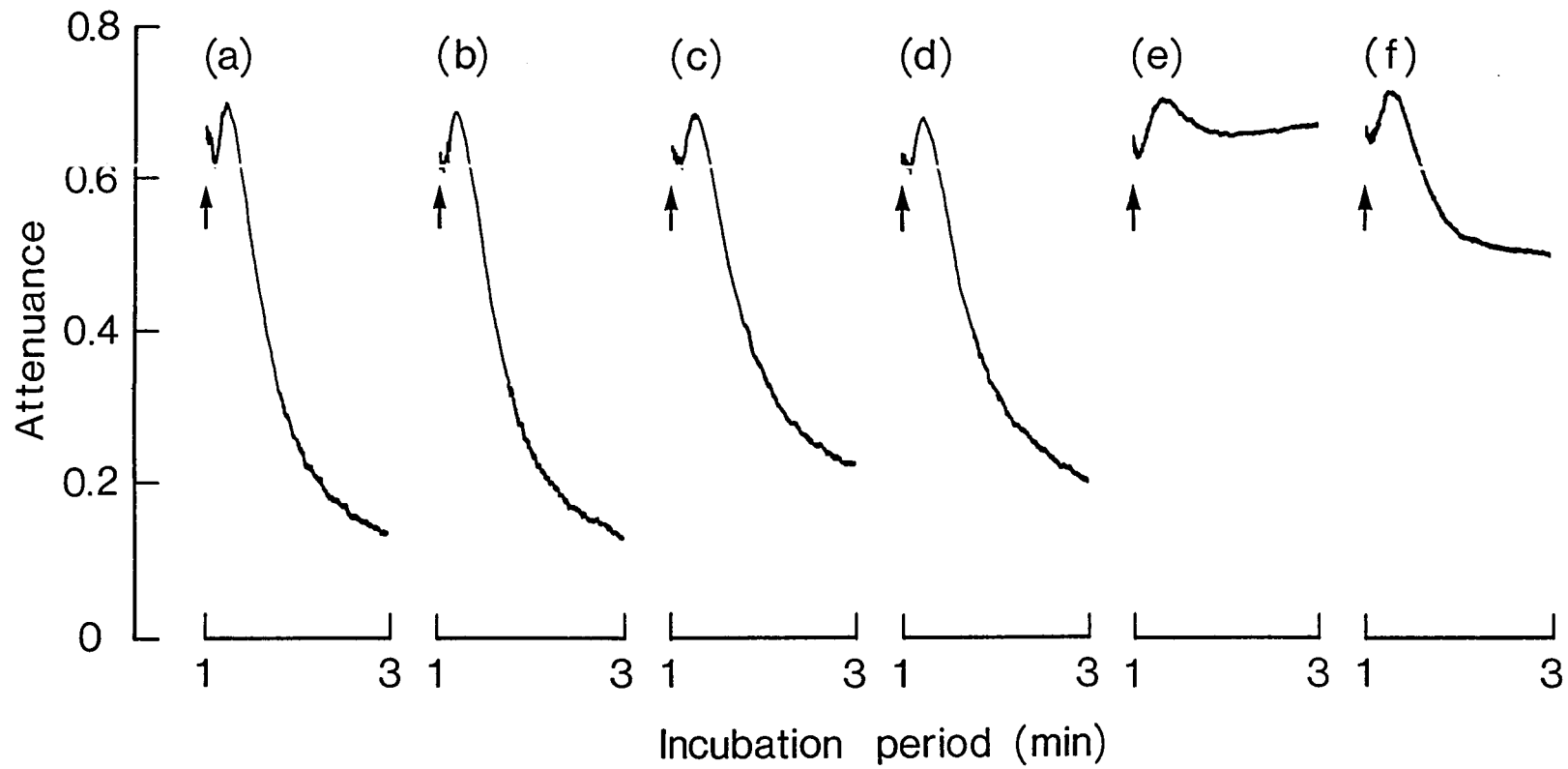
Table 2:   Effects of DDA on the accumulation of cyclic nucleotides in human

platelets caused by NP and EHNA

Platelet metabolic nucleotide pools were prelabelled by incubation with [³H]adenine and

[³H]guanine as described in the Experimental section. Samples of platelet suspension

were incubated for 1 min at 37 °C in the absence or presence of 100 μM DDA and either

no further additions, 10 μM NP, 20 μM EHNA or 10 μM NP + 20 μM EHNA, as

indicated. Platelet [³H]cAMP and [³H]cGMP were isolated and expressed as percentages

of the total platelet [³H]ATP (1.8×10^6 dpm/sample) and [³H]GTP (3.1×10^6

dpm/sample), respectively. Results are the means ± S.E.M. of triplicate measurements in

a single experiment.

Additions	[³H]cAMP (% of [³H]ATP)	[³H]cGMP (% of [³H]GTP)
None	0.025 ± 0.001	0.009 ± 0.002
DDA	0.023 ± 0.001	0.012 ± 0.003
NP	0.041 ± 0.001	0.389 ± 0.006
NP + DDA	0.034 ± 0.001	0.427 ± 0.032
EHNA	0.025 ± 0.001	0.008 ± 0.001
EHNA + DDA	0.026 ± 0.001	0.008 ± 0.001
NP + EHNA	0.055 ± 0.002	0.397 ± 0.012
NP + EHNA + DDA	0.041 ± 0.001	0.457 ± 0.018

increase was much smaller when DDA was present in that $41 \pm 9\%$ of the NP-induced

increase was blocked by DDA (mean $\pm$ S.E.M., 3 expts.). The much larger increase in

[³H]cAMP accumulation induced by EHNA and NP was more potently inhibited by DDA

($83 \pm 7\%$ of the increase caused by NP with EHNA was blocked by DDA, mean $\pm$

S.E.M., 3 expts.).

Since DDA decreased the accumulation of [³H]cAMP in the presence of NP

or NP + EHNA, while exerting no inhibitory effect on [³H]cGMP accumulation, it is the

reduction by DDA of cAMP levels, and not those of cGMP, that attenuates the inhibition

of aggregation observed in the presence of NP with EHNA. These results suggest that it

is the hydrolysis of cAMP by PDE2, rather than of cGMP, that is the more significant in

platelets.

3.4. Comparison of the effects of EHNA and of coformycin

EHNA was originally described as an inhibitor of adenosine deaminase

(Schaeffer and Schwender, 1974). Because inhibition of this enzyme could eventually

lead to the accumulation of adenosine, which can stimulate platelet adenylyl cyclase

(Haslam and Rosson, 1975), it was conceivable that some of the effects of EHNA on

platelet [³H]cAMP levels described in this work were due to the action of EHNA on

adenosine deaminase rather than on PDE2. To address this issue, the effects of EHNA on

platelet cyclic nucleotide levels and on thrombin-induced platelet aggregation were

compared to those of coformycin, a particularly potent inhibitor of adenosine deaminase

(K_i 2.5 to 15 pM) (Bessodes *et al.*, 1982).

Prelabelled platelets were incubated with either no adenosine deaminase

inhibitor, EHNA (20 µM) or coformycin (20 µM), in each case in the presence or absence

of 100 µM NP (Figure 14). This concentration of NP was chosen because the largest

effects of EHNA had been observed in its presence. NP caused very large but somewhat

variable increases in [^{3}H]cGMP accumulation (a range of [^{3}H]cGMP values from 0.6 to

1.1% of total [^{3}H]GTP per sample was observed over three separate experiments).

Coformycin did not significantly change [^{3}H]cGMP accumulation in the presence of NP

($P > 0.05$, 2-sided paired t-test). The increases in [^{3}H]cGMP in the presence of both NP

and coformycin were significantly smaller than those observed with NP and EHNA

($P < 0.05$, 2-sided paired t-test). A slight but consistent increase in [^{3}H]cAMP levels was

observed with coformycin and NP, compared to NP alone (an average increase of 12%

was observed in three separate experiments, $P < 0.05$, 2-sided paired t-test). This

increase in [^{3}H]cAMP accumulation was significantly smaller than that observed with NP

and EHNA ($P < 0.05$, 2-sided paired t-test). The results indicate that very little of the

action of EHNA on platelet [^{3}H]cAMP is attributable to its inhibition of adenosine

deaminase.

Further support for this conclusion was found when the effects of EHNA and

coformycin on platelet aggregation were compared (Figure 15). Neither EHNA (20 µM)

Figure 14: **Comparison of the effects of EHNA and of coformycin on NP-induced cyclic nucleotide accumulation in human platelets**

Platelet metabolic nucleotide pools were prelabelled by incubation with [³H]adenine and [³H]guanine as described in the Experimental section. Samples of platelet suspension were incubated for 1 min at 37 °C in the absence (hatched) or presence (solid) of 100 μM NP and either no further addition (control), 20 μM EHNA, or 20 μM coformycin, as indicated. Platelet [³H]cAMP **(a)** and [³H]cGMP **(b)** were isolated and expressed as percentages of total platelet [³H]ATP (1.3 x 10⁶ dpm/sample) and [³H]GTP (3.6 x 10⁶ dpm/sample), respectively. Results are the means ± S.E.M. of triplicate measurements in a single experiment.
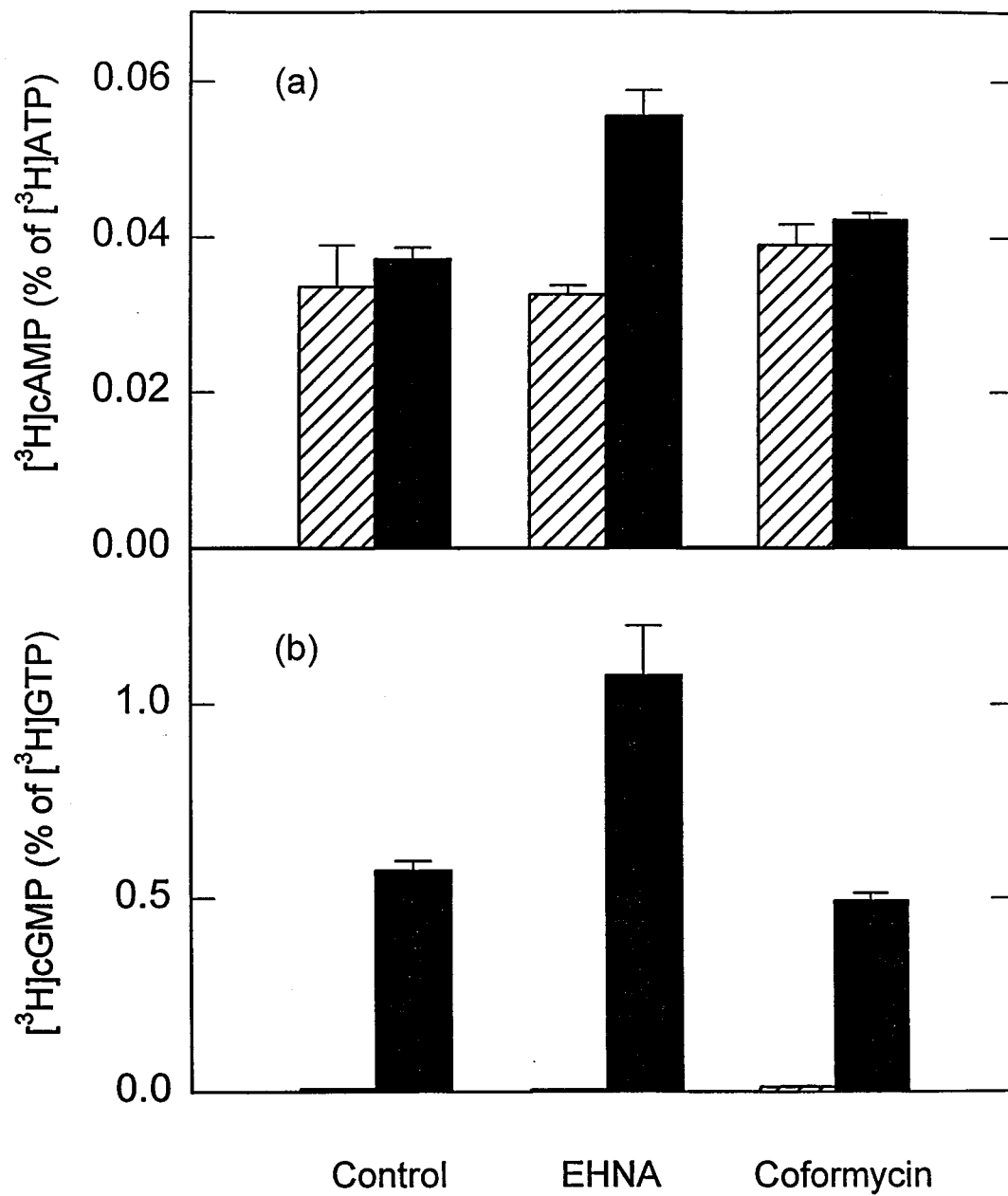
Figure 15: **Comparison of the effects of EHNA and of coformycin on thrombin-induced platelet aggregation**

Human platelets were isolated and washed as described in the Experimental section. Samples of platelet suspension were stirred for 1 min at 37 °C with either no other addition (control), 20 μM EHNA, or 1 μM coformycin, in each case in the absence (**a**) or presence (**b**) of 10 μM NP, as indicated in the figure. Platelet aggregation was then initiated by the addition of human thrombin (final concentration 0.15 i.u./ml, ↓). Tracings of the recordings are shown.
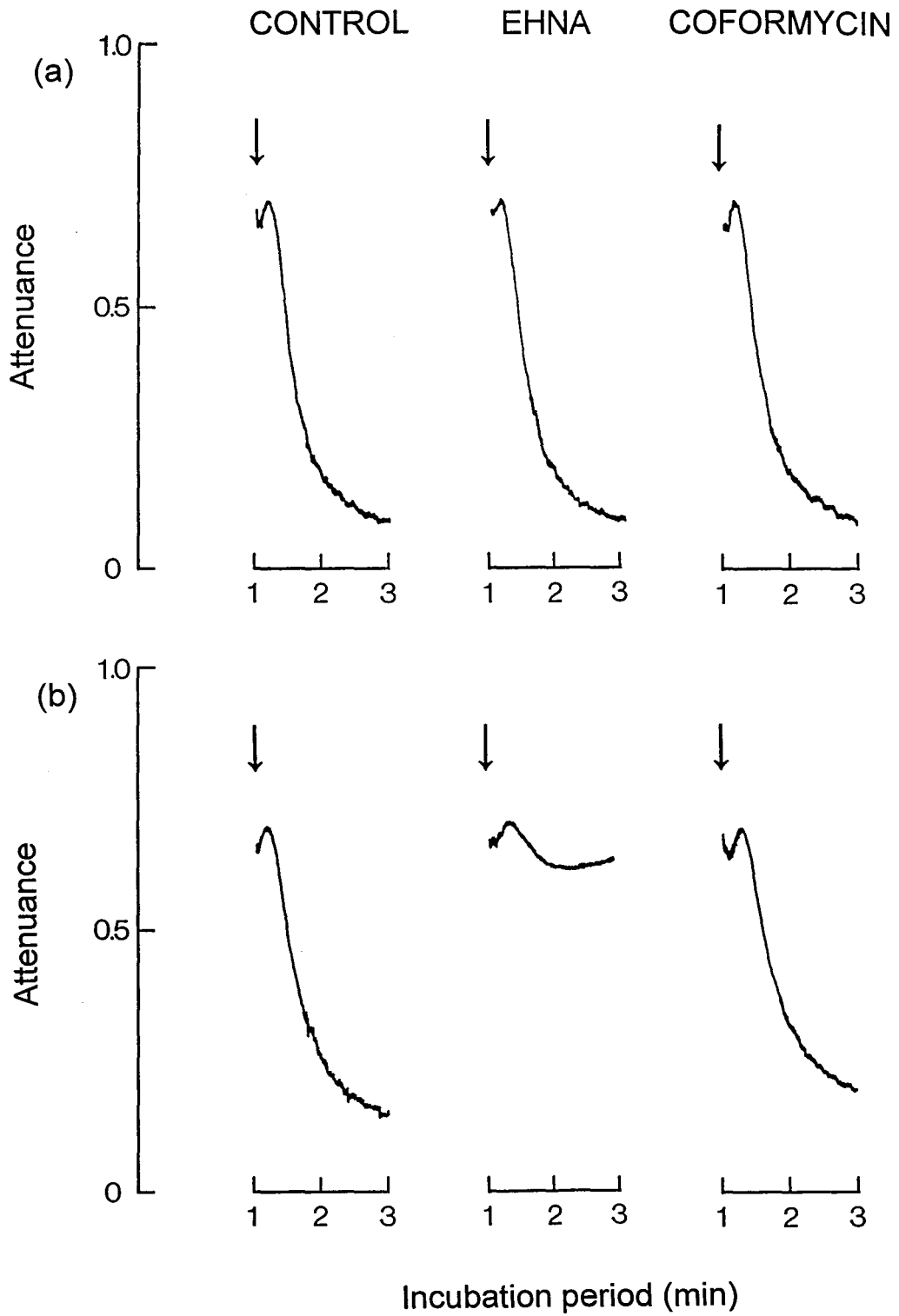
nor coformycin (1 μM) alone inhibited thrombin-induced platelet aggregation. EHNA clearly exerted a strong potentiating effect on the weak inhibition of aggregation caused by NP (10 μM), whereas coformycin had little effect on the action of NP (Figure 15).

Thus, coformycin did not mimic the effects of EHNA on either cyclic nucleotide accumulation or on thrombin-induced platelet aggregation and no evidence was found for major involvement of adenosine deaminase in the effects of EHNA on cyclic nucleotide levels described in this work. It was therefore concluded that the effects of EHNA are due to inhibition of PDE2 and not adenosine deaminase.

3.5. The role of PDE2 in the effects of NP in the presence of low and high platelet cAMP levels

The two platelet cAMP phosphodiesterases, PDE2 and PDE3, differ in their respective substrate affinities; the K_m of PDE3 for cAMP is quite low (0.4 μM; Tang *et al.*, 1997), whereas that for PDE2 it is much higher (50 μM in the absence of cGMP; Manganiello *et al.*, 1990b; Grant and Colman, 1990). PDE3 has been established as a major platelet cAMP phosphodiesterase at low cAMP concentrations, such as in the resting platelet (Shen and Colman, 1995a), but the regulation of higher platelet cAMP levels by PDE2 and PDE3 had not yet been studied. The next question addressed in this research was whether cAMP hydrolysis by PDE2 and PDE3 is also influenced by both the cAMP concentration and the presence of cGMP in intact platelets. An activator of

Table 3: **Effects of a low PGI$_2$ concentration and NP on platelet cyclic nucleotides in the absence or presence of EHNA**

Metabolic nucleotide pools were prelabelled by the incubation of platelets with [^{3}H]adenine and [^{3}H]guanine as described in the Experimental section. Samples of platelet suspension were incubated for 1 min at 37 °C with 20 μM EHNA, 1 nM PGI$_2$ and 10 μM NP in the combinations indicated. Platelet [^{3}H]cAMP and [^{3}H]cGMP were isolated and expressed as percentages of total platelet [^{3}H]ATP (1.8 x 10^6 dpm/sample) and [^{3}H]GTP (2.1 x 10^6 dpm/sample), respectively. Results are the means ± S.E.M. of triplicate measurements in a single experiment.

Additions	[^{3}H]cAMP (% of [^{3}H]ATP)	[^{3}H]cGMP (% of [^{3}H]GTP)
None	0.023 ± 0.004	0.004 ± 0.001
EHNA	0.028 ± 0.000	0.003 ± 0.001
NP	0.036 ± 0.005	0.582 ± 0.021
NP + EHNA	0.065 ± 0.008	0.779 ± 0.042
PGI$_2$	0.045 ± 0.003	0.002 ± 0.002
PGI$_2$ + EHNA	0.039 ± 0.001	0.005 ± 0.002
PGI$_2$ + NP	0.096 ± 0.008	0.559 ± 0.023
PGI$_2$ + NP + EHNA	0.184 ± 0.014	0.834 ± 0.041

platelet adenylyl cyclase, PGI_2, was utilized to increase cAMP levels; increases in cGMP were triggered by NP.

To investigate first the relative roles of PDE2 and PDE3 in regulating lower cAMP levels, prelabelled platelets were incubated with a low concentration of PGI_2 (1 nM) in the presence and absence of NP (10 μM) and EHNA (20 μM) (Table 3). In these experiments (carried out by E. K. Jang), platelet [^{3}H]cGMP levels were increased only in the presence of NP, and these increases were further enhanced by the addition of EHNA, but not by PGI_2. In three separate experiments (including two performed by E. K. Jang), increases in [^{3}H]cAMP accumulation resulted from treatment with either NP (46 ± 12%, mean ± S.E.M.) or PGI_2 (181 ± 47%, mean ± S.E.M.), and a supra-additive increase in [^{3}H]cAMP was observed when both compounds were added simultaneously (456 ± 34%, mean ± S.E.M.). The potentiation of PGI_2-induced [^{3}H]cAMP accumulation by NP is consistent with inhibition of PDE3 by cGMP. Evidence supporting a role for PDE2 in regulating these low levels of cAMP was sought by treating platelets with EHNA (Table 3). In the absence of NP, EHNA did not increase [^{3}H]cAMP levels above those observed in the presence of PGI_2 alone, showing that PDE2 was not active at these low cAMP concentrations.

The supra-additive increases in [^{3}H]cAMP accumulation observed when PGI_2 and NP were added simultaneously were greatly enhanced when EHNA was also present, showing that the action of PDE2 could restrict this synergism. These results are

consistent with the conclusion that the supra-additive increases in [^{3}H]cAMP levels

observed when PGI$_2$ and NP are added simultaneously are mediated through inhibition of

PDE3 and involve PDE2 only as a negative regulator. Unless NP (or cGMP) is present,

PDE2 does not appear to play a major role in regulating low concentrations of cAMP in

platelets.

To determine whether the same conclusions apply when cAMP levels are

high, prelabelled platelets were incubated with a much higher PGI$_2$ concentration

(20 nM) (Figure 16). The effect of cGMP, which was increased by NP (10 μM), on the

relative contribution of each platelet PDE was then assessed by adding selective PDE

inhibitors (Figure 16). As before, significant increases in [^{3}H]cGMP accumulation were

only observed in the presence of NP. In these conditions, inhibition of PDE2 by EHNA

resulted in larger increases in [^{3}H]cGMP accumulation than were caused by inhibition of

PDE5 (the cGMP-selective PDE) by zaprinast. The difference between [^{3}H]cGMP

accumulation observed in the presence of EHNA with NP and that observed in the

presence of zaprinast and NP was variable, and ranged from 0.27 to 1.20% of platelet

[^{3}H]GTP in three separate experiments. Addition of 20 nM PGI$_2$ alone caused a very

large increase in platelet [^{3}H]cAMP (50 ± 5-fold, mean ± S.E.M., 3 expts.). In contrast to

the lack of effect observed with a low PGI$_2$ stimulus (Table 3), the addition of EHNA in

this case resulted in a further increase in [^{3}H]cAMP accumulation (an average increase of

83 ± 8%, mean ± range, in 2 expts.), greater than that observed when PDE3 was inhibited

by lixazinone ($34 \pm 3\%$, mean $\pm$ S.E.M., 3 expts.). Inhibition of PDE5 had no effect on

[³H]cAMP accumulation. These results show that in the presence of high intracellular

concentrations of cAMP, PDE2 becomes an important phosphodiesterase activity,

whether cGMP is present or not. In contrast to the supra-additive increases in [³H]cAMP

that were observed when 10 µM NP and 1 nM PGI_2 were added together (Table 3), in the

presence of 20 nM PGI_2 the net effect of NP was a significant *decrease* in [³H]cAMP

levels ($P < 0.05$, 2-sided paired *t*-test, 3 expts.) (e.g. Figure 16, "control"). This effect

was only blocked by inhibiting PDE2 with EHNA.

As a whole, these studies reveal much about the relative roles of PDE2 and

PDE3. The predominance of PDE3 in the regulation of low cAMP levels was confirmed

when cGMP was absent, but PDE2 became the major PDE when cGMP was present.

The results are consistent with a model in which there are two cues that determine which

enzyme is active: the presence or absence of NP (or cGMP), and the concentration of

cAMP. When PDE2 is activated by cGMP, it plays a major role in the regulation of

platelet cAMP levels. At low cAMP levels, when PDE3 is the major cAMP

phosphodiesterase, the net effect of NP (or cGMP) is to cause an increase in cAMP by

inhibiting PDE3, but at higher cAMP levels the activity of PDE2 is much increased

relative to that of PDE3, and the net effect of NP (or cGMP) on PDE2 outweighs its

effect on PDE3, resulting in a decrease in cAMP.

Figure 16: Inhibition by NP of the increase in platelet [^{3}H]cAMP caused by

20 nM PGI$_2$; suppression of this effect by EHNA

Metabolic nucleotide pools were prelabelled by incubation of platelets with [^{3}H]adenine

and [^{3}H]guanine as described in the Experimental section. Samples were incubated for

1 min at 37 °C with 20 nM PGI$_2$ in the absence (hatched columns) or presence (solid

columns) of 100 μM NP and other additions as follows: none (control); 20 μM EHNA;

10 μM zaprinast (Zap); 1 μM lixazinone (Lix); 20 μM EHNA + 10 μM zaprinast; 20 μM

EHNA + 1 μM lixazinone. Platelet [^{3}H]cAMP (**a**) and [^{3}H]cGMP (**b**) were isolated and

expressed as percentages of the total [^{3}H]ATP (1.3 x 10^6 dpm/sample) and [^{3}H]GTP

(3.6 x 10^6 dpm/sample), respectively. Results are the means ± S.E.M. of triplicate

measurements in a single experiment.
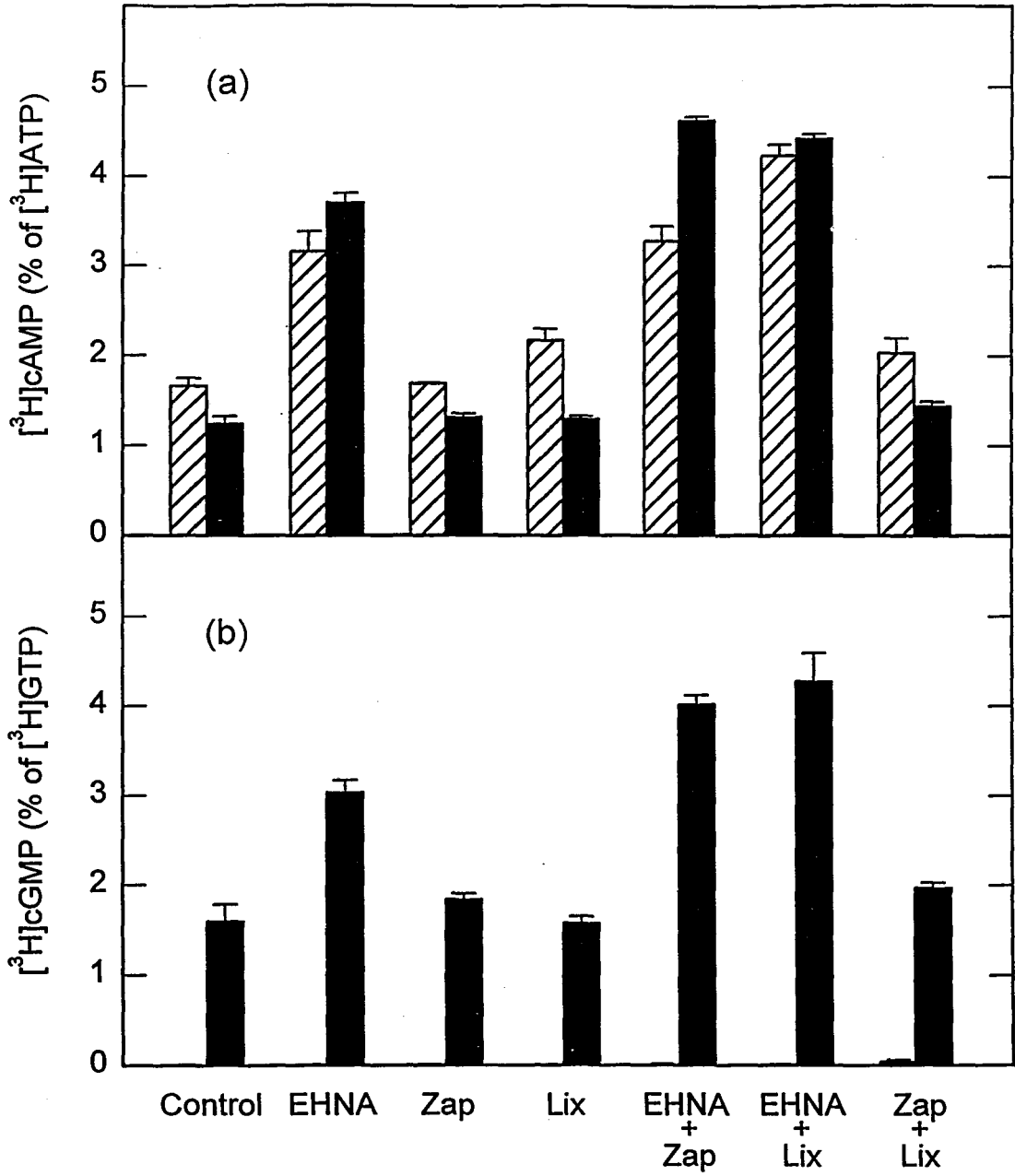
3.6. Role of guanylyl cyclase in NP-induced changes in platelet cyclic nucleotide levels

Many of the conclusions derived from these studies depend upon the assumption that NP exerts its effects on platelet cyclic nucleotides through its ability to stimulate soluble guanylyl cyclase. However, NP releases NO, which is a highly reactive molecule that could act on targets other than guanylyl cyclase (Star, 1993). To ascertain if NP has actions that are independent of guanylyl cyclase, its effects on platelet cyclic nucleotides were determined in the presence of the guanylyl cyclase inhibitor ODQ (Figure 17). Prelabelled human platelets were incubated with EHNA (20 μM), lixazinone (1 μM) and a range of NP concentrations (0 to 100 μM), in each case in the absence or presence of ODQ (10 μM) (Figure 17). Inhibition of guanylyl cyclase with 10 μM ODQ blocked $92 \pm 1\%$ of the increase in [^{3}H]cGMP caused by 10 μM NP (Figure 17). As a result, the biphasic effect of NP on [^{3}H]cAMP accumulation (Figure 9) was completely prevented, presumably because there was no longer any inhibition of PDE3 by cGMP. Moreover, ODQ blocked activation of PDE2 by NP. In earlier experiments, NP was shown to overcome the effects of inhibition of PDE3 (with lixazinone) by activating PDE2 (Figure 9). When these conditions were tested again in the presence of ODQ, the inhibitory effect of NP was abolished (Figure 17), showing that NP depends on cGMP to activate PDE2. All of the actions of NP on platelet cyclic nucleotide levels that had been observed earlier were abolished by inhibition of guanylyl cyclase, showing that the

Figure 17: **Effects of ODQ on NP-induced changes in platelet cyclic nucleotide**

levels in the presence of lixazinone and EHNA

Platelet metabolic nucleotide pools were prelabelled by incubation with [³H]adenine and

[³H]guanine. Samples of labelled platelet suspension were incubated for 1 min at 37 °C

with the indicated concentrations of NP in the absence (hollow symbols) or presence

(filled symbols) of the guanylyl cyclase inhibitor ODQ (10 μM) and other additions as

follows: none (O, ●); 20 μM EHNA (to inhibit PDE2) (□, ■); 1 μM lixazinone (to

inhibit PDE3) (∇, ▼). Platelet [³H]cAMP (**a**) and [³H]cGMP (**b**) were measured and are

expressed as percentages of total [³H]ATP (6×10^5 dpm/sample) and [³H]GTP

(1.2×10^6 dpm/sample), respectively. Results are the means ± S.E.M. of triplicate

measurements in a single experiment.
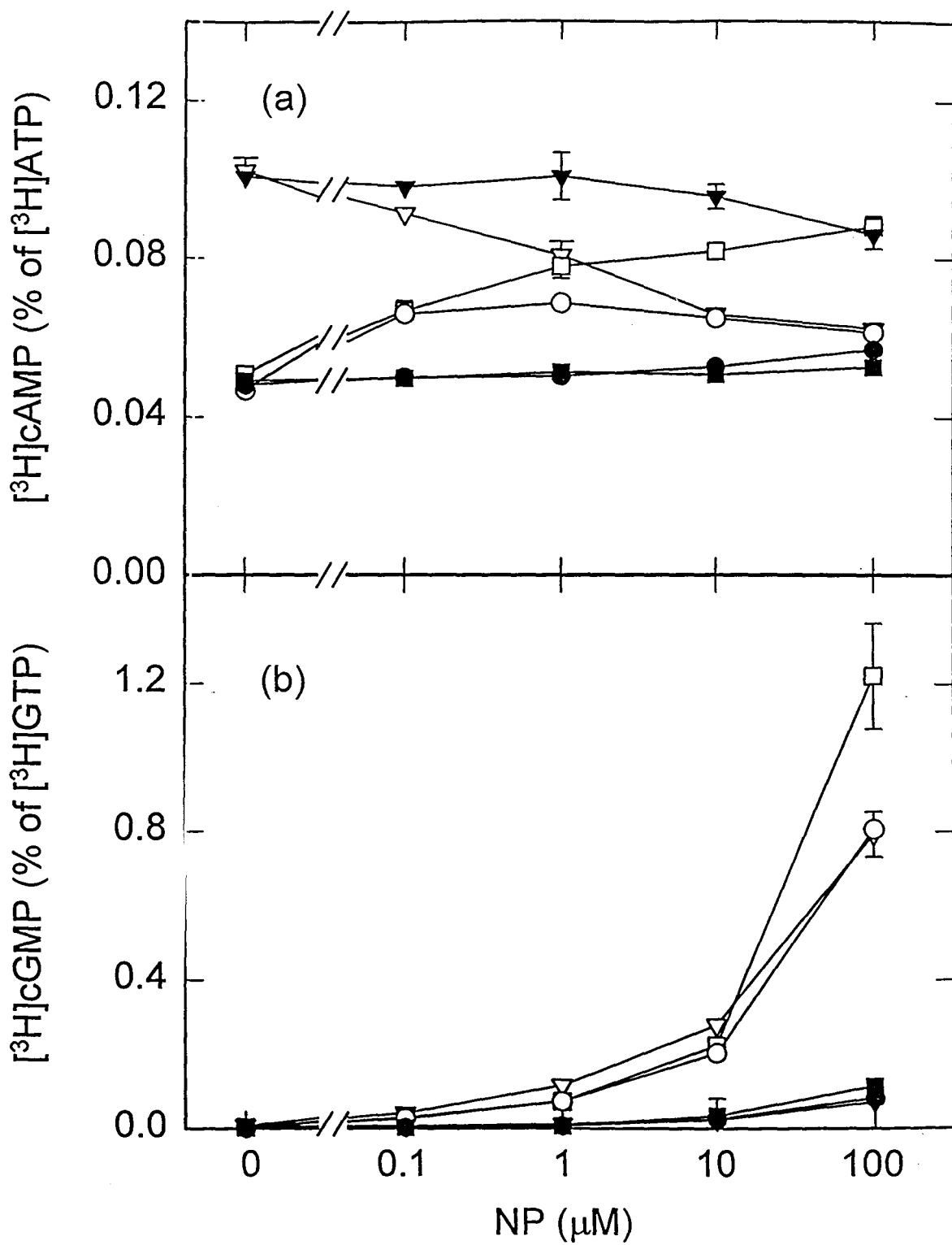
effects of NP on platelet cAMP depend upon cGMP formation.

3.7.   Studies on collagen-induced platelet aggregation

Like thrombin, collagen is a strong activator of platelets. Although the

primary mechanism of collagen action is through the $cPLA_2$ pathway, leading to

production of TXA_2, activation of guanylyl cyclase also accompanies collagen-induced

platelet aggregation (Davies *et al.*, 1976). Since many experiments in this study have

demonstrated activation of PDE2 by cGMP and have shown that activation of PDE2 has

important consequences for thrombin-induced platelet aggregation, it was important to

determine whether collagen-induced increases in cGMP also lead to activation of PDE2.

In these studies, formation of cGMP was prevented by ODQ. The effects of ODQ on

collagen-stimulated platelet aggregation in the presence of NP and EHNA are presented

in Figure 18. When added alone, EHNA did not inhibit collagen-induced aggregation,

suggesting that if collagen does increase cGMP, it is either insufficient to stimulate PDE2

activity or any stimulation of PDE2 is without functional consequences (data not shown).

A partial inhibitory effect of NP (10 µM) was observed (Figure 18, **a** and **b**), and was

strongly potentiated by EHNA (20 µM) (Figure 18 **c**). ODQ blocked all the inhibitory

effects of NP plus EHNA on collagen-induced platelet aggregation (Figure 18 **d**). The

synergistic effects of NP and EHNA were similar to the results obtained in the case of

thrombin-induced platelet aggregation (Figure 11), indicating that activation of PDE2

Figure 18:   **Effects of NP, EHNA and ODQ on collagen-induced platelet**

                       aggregation

Human platelets were isolated and washed as described in the Experimental section.

Samples of platelet suspension (0.45×10^8 platelets in total) were stirred at 37 °C with

collagen (30 µg/ml) in the presence of the following other additions: **a**, none; **b**, 10 µM

NP; **c**, 10 µM NP + 20 µM EHNA; **d**, 10 µM NP + 20 µM EHNA + 10 µM ODQ. In

each case, these compounds were mixed with the platelets at the same time as the

collagen. All incubations then proceeded for a further 2 min during which aggregation

was recorded. Addition of 20 µM EHNA or 10 µM ODQ alone had no effect (data not

shown).
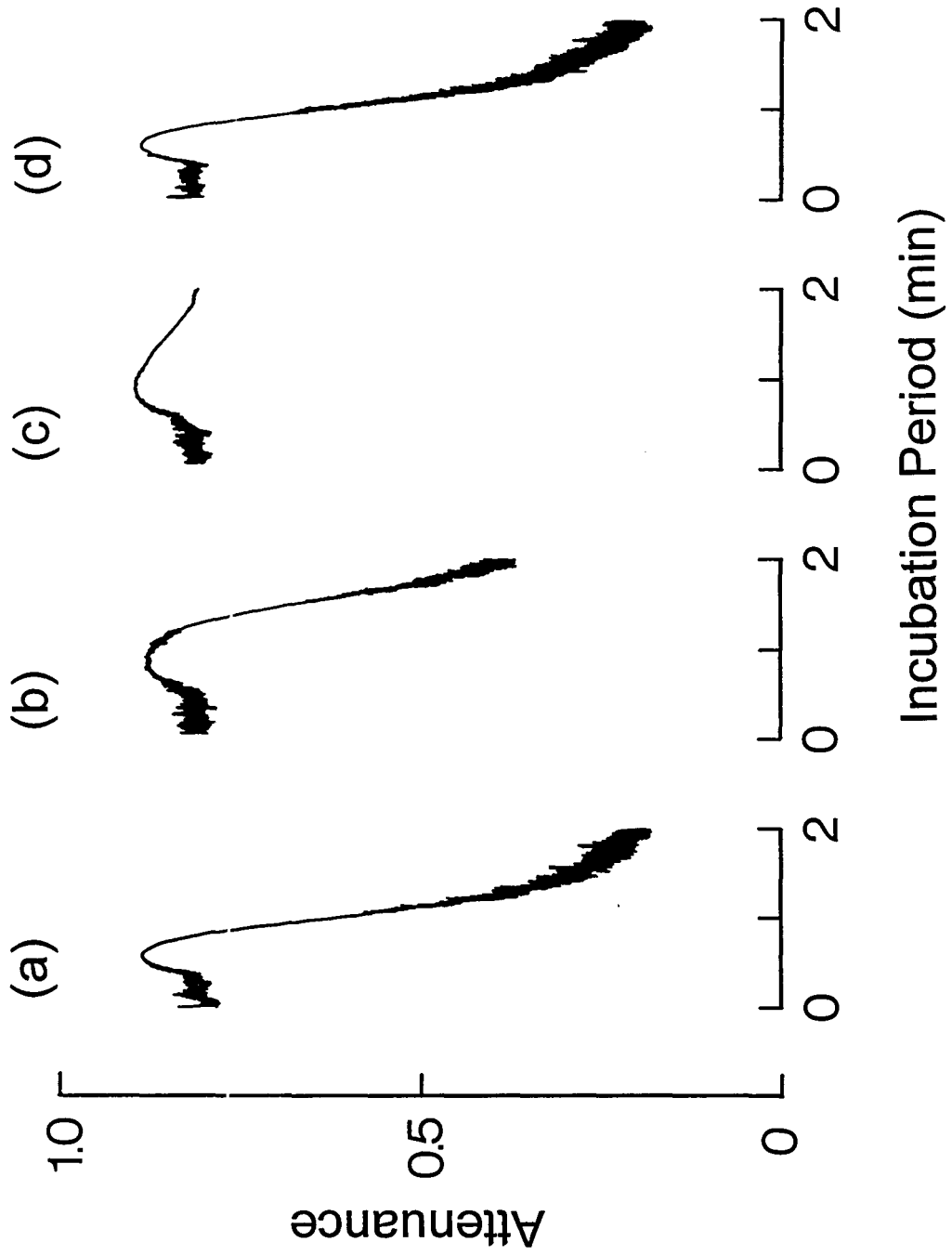
limits the inhibitory effects of NP on collagen-induced platelet aggregation.

In order to observe the potentiation by EHNA of the inhibitory effects of NP, it was crucial to establish the appropriate experimental conditions. The degree of inhibition exerted by NP was highly dependent on the strength of the collagen stimulus; as the concentration of collagen was increased, the effect of NP was progressively reduced from a complete inhibition of aggregation to a partial inhibitory effect (Figure 19). In conditions in which aggregation was prevented by NP, as in the presence of 10 or 20 μg of collagen/ml (Figure 19), any potentiating effect of EHNA was masked by the effect of NP alone.

It has been reported that collagen-induced aggregation is potentiated by ODQ, suggesting that stimulation of guanylyl cyclase by collagen exerts an inhibitory effect on aggregation (Moro *et al.*, 1996). To investigate whether this reported effect could be confirmed, the effects of ODQ on collagen-induced aggregation were therefore investigated. In these experiments, the amount of collagen was adjusted to result in a partial stimulation of aggregation. In these conditions, ODQ did not exert a strong potentiating effect (data not shown). A slight potentiating effect was observed in two separate experiments, whereas in two other experiments no effect of ODQ was observed.

Figure 19: **The effect of the strength of the collagen stimulus on inhibition of**

platelet aggregation by NP

Human platelets were isolated and washed as described in the Experimental section.

Samples of platelet suspension (0.45×10^8 platelets in total) were incubated for 2 min at

37 °C whilst stirring with collagen in the presence and absence of 10 μM NP. The final

concentration of collagen was 10, 20, or 30 μg/ml, as indicated in the figure. Tracings of

the recordings are shown.
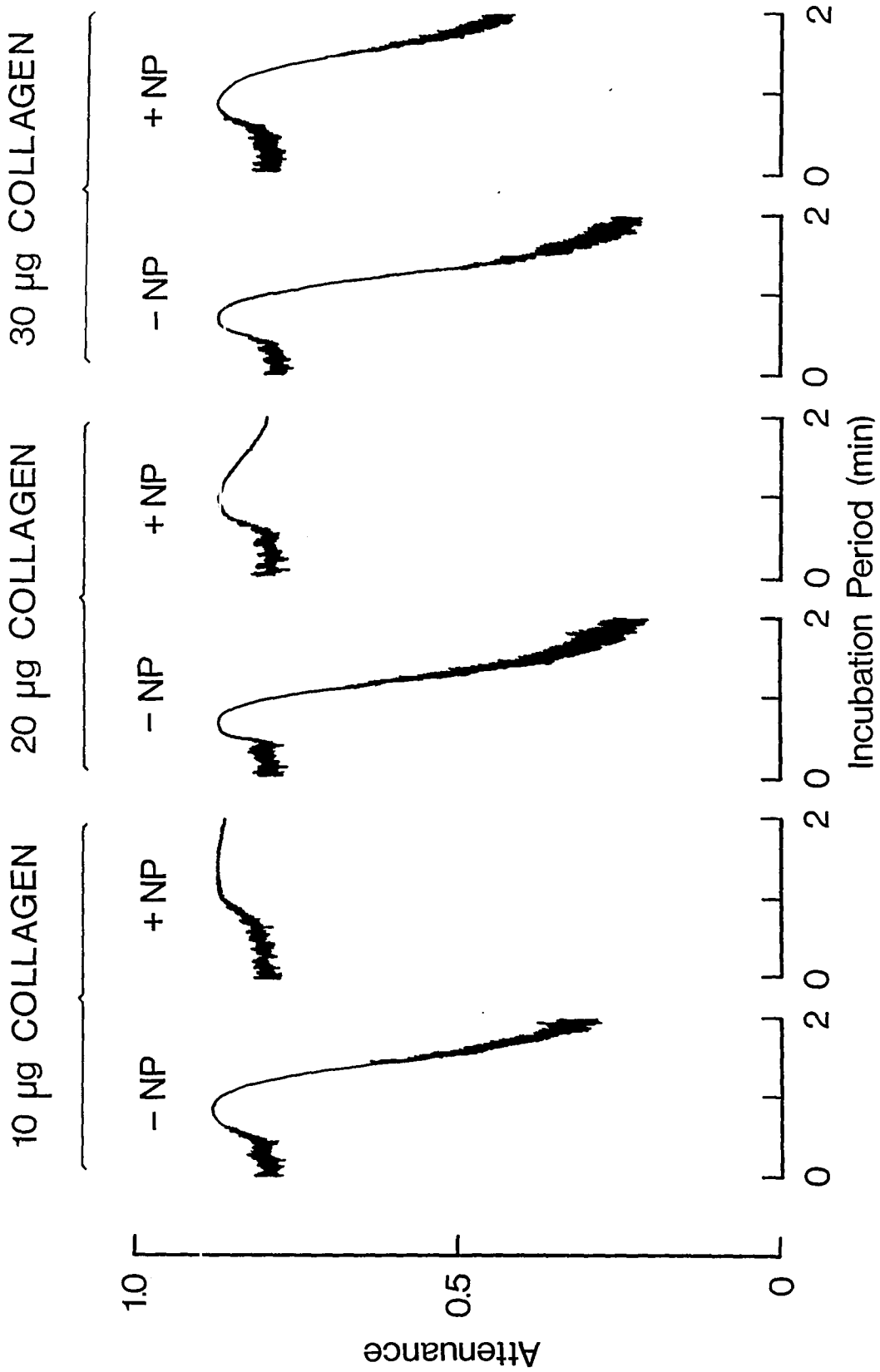
4.   DISCUSSION

4.1.   Introduction

The goal of my research has been to investigate the role of PDE2 in human

platelets. Although it is well-established that PDE3 is the major cAMP hydrolysing

activity in resting platelets (Sheth and Colman, 1995), the contribution of PDE2 to the

regulation of platelet cyclic nucleotide levels and platelet responses had not yet been

addressed. In the present study, the effects of PDE2 on cyclic nucleotide accumulation

were therefore investigated in platelets that were treated with activators of adenylyl

and/or guanylyl cyclases. These studies were paired with experiments on thrombin- and

collagen-induced platelet aggregation, to determine how the observed effects of PDE2 on

cyclic nucleotide levels modulated platelet responses. The results show that in the

presence of cGMP or when cAMP levels are high, PDE2 plays a major role in regulating

platelet cyclic nucleotide levels and platelet function.

4.2.   Effects of EHNA on partially purified PDE2

EHNA has only recently been reported to inhibit PDE2 activity (Podzuweit *et*

al., 1995). At the time that my investigations were initiated, there was no published

information concerning the effects of EHNA on platelet PDE2.

My research shows that inhibition of PDE2 by EHNA is selective when

compared with its effects on the activities of the other PDEs that are present in human platelets. This conclusion is supported by studies performed with PDEs isolated from other tissues. In both rat hepatocytes and in myocardium, in which PDEs 1 through 4 are present, inhibition by EHNA has been shown to be selective for PDE2 (Michie *et al.*, 1996).

In some of my experiments, a weak stimulation of cAMP hydrolysis by low concentrations of EHNA was observed. This effect of EHNA could underlie its observed ability to reduce the inhibitory effect of lixazinone on platelet aggregation. Stimulation by EHNA was most pronounced when the compound was present at concentrations between 1 and 5 μM. In a recent publication, stimulation of PDE2 activity by EHNA was evident, although it was not remarked upon by the authors (Michie *et al.*, 1996). These authors examined the effects of EHNA on cGMP-stimulated PDE2 from platelets and thymocytes. Although no stimulatory effect of EHNA was observed with the platelet enzyme, the activity of PDE2 from thymocytes was markedly enhanced by 0.1 to 0.5 μM EHNA. Thus, stimulation by low concentrations of EHNA has been observed with PDE2 isolated from other tissues, but the effect was variable.

A similar effect has been reported for 1-isobutyl-3-methylxanthine (IBMX), which at lower concentrations weakly stimulates PDE2 activity but at higher concentrations acts as an inhibitor (Stroop and Beavo, 1991). A possible explanation for the stimulatory effect of IBMX is that when this agent binds to the catalytic site on one

half of the PDE2 dimer it induces cooperative conformational changes in the other catalytic site (Stroop and Beavo, 1991). It is possible that the observed stimulatory effects of EHNA arise from a similar mechanism.

The stimulatory effect of EHNA was variable, and ranged from undetectable to quite pronounced. Before any studies on the mechanism underlying the stimulatory effect of EHNA can be initiated, the conditions in which this effect consistently occurs must be fully defined. Experiments are required to outline the concentrations of EHNA that exert stimulatory effects, and also to examine why this effect of EHNA appears to differ with respect to hydrolysis by of cAMP and cGMP by PDE2.

In this research, other experiments were performed in a preliminary attempt to understand the mechanism of the inhibitory action of EHNA. It was observed that activation of cAMP hydrolysis by cGMP was inhibited in the presence of EHNA, an effect that was also noted by Michie et al. (1996) in their studies on platelet PDE2. Furthermore, in both the present investigation and in that of Michie et al. (1996), the inhibitory effects of EHNA on cAMP hydrolysis by PDE2 were more pronounced in the presence of cGMP. Because PDE2 binds cyclic nucleotides at both regulatory and active sites and the structure of EHNA bears some similarity to that of adenine or cAMP, it is possible that EHNA could be exerting effects at either of these sites. However, in the present study, when the inhibition of cAMP hydrolysis by EHNA was examined over a range of cGMP concentrations, the percentage of cGMP-stimulated activity inhibited by

EHNA was not affected by cGMP. This suggests that cGMP and EHNA are not acting

on the same site on PDE2. There are several models which are consistent with these

observations, models which involve either intra- or inter- subunit interactions in PDE2.

There are two non-catalytic cGMP binding sites per homodimer of PDE2 (Stroop and

Beavo, 1991). It may be that binding of cGMP to a regulatory site of PDE2 induces

intra-subunit and/or inter-subunit conformational changes in the active sites which favour

binding of EHNA and thereby increases the inhibition exerted by EHNA on catalysis. It

is also possible that binding of cGMP to one regulatory site may favour binding of EHNA

to the other regulatory site in the dimer, where EHNA could exert inhibitory effects.

Although there have been studies performed on the mechanism of action of

IBMX on PDE2 (Stroop and Beavo, 1991, and the references therein), detailed studies on

the mechanism of action of EHNA are lacking. Such information would be quite

instructive with respect to PDE2, because EHNA is the only known selective inhibitor of

this enzyme. A definitive analysis of the action of EHNA on PDE2 would require more

highly purified enzyme, a goal which was not attainable in the present study. However, if

such a preparation were obtained one could perform a detailed kinetic analysis in which

K_i measurements would be made for EHNA with respect to cAMP and cGMP substrates.

Furthermore, binding studies are required to determine the site(s) with which EHNA

interacts, and whether binding is affected by cGMP. A great deal of exciting research

remains to be done in this area; this will increase our understanding not only of EHNA

but also of PDE2.

4.3.   Prelabelling assays for platelet cyclic nucleotides

The prelabelling method is a sensitive technique for the detection of changes in

platelet cyclic nucleotide levels. The merit of this method has been established in several

ways (Maurice and Haslam, 1990a; Maurice *et al.*, 1993). First, the identities of the

[^{3}H]cAMP and [^{3}H]cGMP peaks eluted after Dowex chromatography were established by

performing TLC on eluted samples to which authentic [^{14}C]cAMP or [^{14}C]cGMP,

respectively, had been added. Almost all of the [^{3}H]cAMP co-chromatographed with

[^{14}C]cAMP. Between 40 and 100% of the basal [^{3}H]cGMP migrated with [^{14}C]cGMP,

which may be explained by the very low level of cGMP measured in unstimulated

platelets. However, any increases in [^{3}H]cGMP were shown to be authentic cGMP.

The accuracy of the prelabelling method in measuring platelet cyclic

nucleotides was tested by comparison with radioimmunoassays (Maurice and Haslam,

1990a). Samples of prelabelled platelets were treated with digitonin to release cytosol,

and nucleotides were then isolated and separated by TLC. Labelled [^{3}H]ATP and

[^{3}H]GTP were scraped from the TLC plates, samples were counted for ^{3}H and the mass

amounts were determined spectrophotometrically. From these values, the specific

activities of the metabolic precursors of the platelet cyclic nucleotides were determined,

and the masses of cyclic [^{3}H]nucleotide/10^9 platelets were calculated for both [^{3}H]cAMP

and [^{3}H]cGMP. These values were compared with those obtained by

radioimmunoassays. The prelabelling method was shown to give results that were not

significantly different from radioimmunoassays, and smaller standard errors were

obtained with the former method (Maurice and Haslam, 1990a). It should be noted that

in the aforementioned comparison, markers were included to correct for recoveries of

cAMP and cGMP in the radioimmunoassays. When this precaution is not taken, as in

work by others who have failed to detect increases in cAMP in platelets treated with NP

(Mellion et al, 1983; Morgan and Newby, 1989; Halbrugge et al., 1990), the errors

associated with radioimmunoassays may be much higher. Finally, prelabelling methods

are inherently more sensitive than radioimmunoassays, in which the relationship between

changes in the concentrations of cyclic nucleotides and the signal obtained is non-linear.

These methodological considerations are of importance in interpretation of the

discrepancies in the literature (see Section 4.4.).

4.4.   Role of PDE2 in the effects of NP on platelet cAMP accumulation

The increases in platelet cAMP caused by NO donors that have been observed

in this laboratory and by some others (Andersson and Vinge, 1991; Grunberg et al., 1995;

Anfossi et al., 1995) have been controversial in the past, because other investigators have

failed to detect any increases in cAMP (Mellion et al., 1983; Halbrugge et al., 1990;

Morgan and Newby, 1989). However, the latter authors measured platelet cyclic

nucleotides using radioimmunoassays, which are subject to larger errors (see Section 4.3.), particularly if no corrections are made for the recoveries of cyclic nucleotides.

In the present experiments, a biphasic effect of NP on cAMP accumulation was demonstrated. The stimulatory effects of guanylyl cyclase activators on cAMP accumulation have been observed repeatedly in human platelets (Haslam *et al.*, 1980; Andersson and Vinge, 1991; Grunberg *et al.*, 1995; Anfossi *et al.*, 1995; Fisch *et al.*, 1995), as well as in rabbit platelets in which this effect was shown to be caused by inhibition of PDE3 by cGMP (Maurice and Haslam, 1990a). At the outset of this investigation, a molecular mechanism underlying the inhibitory component of the effects of NP on cAMP accumulation had not yet been defined. In many separate experiments in the present research, this component was blocked by EHNA. These studies provided the first demonstration that the inhibitory effects of NP on cAMP accumulation involved PDE2, and raised the possibility that this enzyme has important regulatory effects in the platelet.

Because NP is presumed to release NO in platelet cytosol, there existed a remote possibility that NP exerted its effects on platelet cyclic nucleotides through a target of NO other than guanylyl cyclase. However, because the effects of NP on both platelet cAMP and cGMP were abolished by ODQ, which inhibits guanylyl cyclase but not adenylyl cyclase (Garthwaite *et al.*, 1995), it is clear that the effects of NP, including the increased accumulation of cAMP, depended upon activation of guanylyl cyclase.

4.5.   Inhibitory effects of cAMP and cGMP on platelet function

The present research shows that PDE2 restricts the stimulatory effects of NP on the accumulation of cAMP and to a lesser extent of cGMP. To determine whether this increased accumulation of platelet cyclic nucleotides was reflected in changes in platelet responsiveness, the effects of NP and EHNA on thrombin- and collagen-induced platelet aggregation were tested. It was demonstrated that inhibition of PDE2 by EHNA greatly increased the inhibitory effects of NP on platelet aggregation. This shows - for the first time - that PDE2 plays an important role in the regulation of platelet function by NO or NO donors.

PDE2 hydrolyses both cAMP and cGMP. Does the action of PDE2 on one of these substrates cause more significant effects on platelet function than its action on the other? It is well-known that activators of adenylyl cyclases and of guanylyl cyclases are each able to inhibit platelet aggregation (Haslam *et al.*, 1980). The relative importance of cAMP and cGMP in the inhibition of platelet aggregation is, however, a contentious issue. One group has maintained that the inhibitory effects of these cyclic nucleotides result from separate inhibitory pathways for cAMP and cGMP (Alheid and Förstermann, 1989). However, regulation of cAMP hydrolysis by cGMP implies that the inhibitory effects of these cyclic nucleotides are not independent and that there may be cross-talk at the second-messenger level as well as between PKA and PKG. One of the classical tests of the importance of a second messenger in a response is to prevent its

formation/accumulation and then observe the ensuing effects. However, it was not possible in the present study to prevent the formation of cGMP alone, because when increases in cGMP were blocked with ODQ, the effects of cGMP on cAMP accumulation (through PDE3 and PDE2) were concomitantly suppressed. Therefore, only the role of cAMP in platelets can be directly tested.

EHNA enhanced both the inhibitory effect of NP on platelet aggregation and the stimulatory effect of NP on the accumulation of both cyclic nucleotides. Which cyclic nucleotide is responsible for the potentiation of inhibition? To address this issue, platelets were treated with DDA, an inhibitor of adenylyl cyclase. When the extent of cAMP accumulation was reduced by DDA, a corresponding reduction in the inhibition of platelet aggregation by NP plus EHNA was observed. DDA did not inhibit cGMP accumulation. This suggests that when activation of PDE2 opposes the inhibition of platelet aggregation, it is the action of PDE2 on cAMP rather than cGMP which has the greater effect.

In many other systems, increases in cGMP are associated with a reduction in cAMP concentration. For example, in coronary endothelium and in PC12 cells, NP stimulates soluble guanylyl cyclase and leads to a reduction in cAMP levels (Hempel *et al.*, 1996; Whalin *et al.*, 1991). In other cases, activation of particulate guanylyl cyclase by ANF decreases cAMP levels (Delporte *et al.*, 1996; MacFarland *et al.*, 1991). The present experiments with EHNA demonstrate a molecular mechanism for the decrease in

cAMP levels caused by cGMP in human platelets, namely activation of PDE2, and suggest that the similar effects of cGMP on cAMP accumulation in other systems may be caused by the same mechanism. The emergence of EHNA as a PDE2-selective inhibitor will aid in defining the role of PDE2 in these other systems.

PDE2 has already been demonstrated to play a regulatory role in several tissues. For example, inhibition of PDE2 with EHNA causes increases in cAMP accumulation which lead to vasodilation in the rat lung (Haynes *et al.*, 1996). Similarly, cGMP is known to inhibit the cAMP-dependent Ca^{2+} current in frog myocytes, and this effect of cGMP was also blocked when PDE2 was inhibited by EHNA (Méry *et al.*, 1995). Thus, inhibition of PDE2 has been shown to prevent the inhibitory effects of higher concentrations of cGMP on cAMP-dependent processes in several systems, in addition to human platelets.

The results of the present research thus reinforce a growing awareness of the importance of PDE2 in regulating cAMP-dependent processes and above all in mediating the inhibitory effects of cGMP on cAMP levels.

4.6.   Effects of PDE2 activity on the inhibition of thrombin-and collagen-induced platelet aggregation by cyclic nucleotides

Because there is such a close connection between increases in platelet cyclic nucleotides and inhibition of platelet responses, a significant component of this research

examined whether the effects of activation of PDE2 were reflected in the functional responses of the platelets. Many of the effects of NP and of PDE2 activation on platelet cAMP levels that were measured in prelabelled platelets correlated with the results of experiments on thrombin- and collagen-induced platelet aggregation. Thus, inhibition of PDE2 by EHNA increased the stimulatory effect of NP on cAMP accumulation and also increased the inhibitory effect of NP on aggregation. Similarly, inhibition of PDE3 by lixazinone increased cAMP accumulation and also inhibited thrombin-induced aggregation. Activation of PDE2 by NP diminished the effects of lixazinone on both cAMP accumulation and on thrombin-induced platelet aggregation. These studies demonstrate that PDE2 is a significant enzyme in human platelets, and that the major effect of its activation is to reduce the inhibition of aggregation by agents that increase platelet cAMP. These results are also significant because they suggest that the activation of PDE2 by cGMP *in vitro* also occurs *in vivo*.

4.7.   Roles of PDE2 and PDE3 at low and high cAMP levels

PDE inhibitors had different effects when platelet adenylyl cyclase was stimulated by low and high concentrations of PGI_2. At low PGI_2 concentrations, EHNA did not further increase cAMP, whereas with high PGI_2 concentrations it did so. This shows that PDE2 is not a major cAMP phosphodiesterase at low cAMP levels when cGMP is absent, conditions that are found in the resting platelet *in vitro*. However, when

cAMP levels are increased manyfold, the activity of PDE2 is at least as great as that of

PDE3. This finding may have physiological relevance when one considers that

stimulated endothelial cells release PGI_2 and that simultaneous release of NO may occur

under many conditions. Thus, one may envision that activation of PDE2 by cGMP

occurs *in vivo*.

The observed effects of NP (or cGMP) on cAMP accumulation depended upon

the level of cAMP. When PGI_2 concentrations were low, NP caused a stimulation of

cAMP accumulation, presumably by inhibition of PDE3. However, when the

concentration of PGI_2 was high, NP caused a decrease in cAMP levels, attributable to

activation of PDE2. The same effect has been reported in human platelets by other

investigators (Fisch *et al.*, 1995). These authors found that the accumulation of cAMP

induced by low concentrations of the adenylyl cyclase activators, PGE_1 and iloprost, were

enhanced when guanylyl cyclase was stimulated by the NO donor , SIN-1. Conversely,

at higher concentrations of PGE_1 or iloprost, SIN-1 caused an inhibition of cAMP

accumulation. Thus, although the inhibitory effect of NP on accumulation of high

concentrations of cAMP has been observed by others, the present investigation is the first

to propose a molecular basis for the observed effect, namely activation of PDE2 by NP.

Therefore, it appears that there are two conditions in which the activity of

PDE2 is prominent, namely in the presence of cGMP or of high levels of cAMP. Both of

these cues are associated with inhibition of platelet activation (Haslam *et al.*, 1980).

Under either condition, this enzyme becomes a major regulator of platelet cyclic

nucleotide concentrations and thus of the inhibition of platelet function.

4.8.   Interactions between the effects of PDE2 and those of PDE3

PDE3 is well-established as the major cAMP phosphodiesterase in resting

platelets. When PDE3 was inhibited by lixazinone, a large increase in cAMP levels and a

corresponding inhibition of thrombin-induced aggregation was observed. Both effects of

lixazinone were reduced when PDE2 was activated by cGMP. This is significant when

one considers that there has been a major effort on the part of the pharmaceutical industry

to target platelet PDE3 with inhibitors, the goal being a down-regulation of platelet

responses and thus the prevention of thrombosis (reviewed by Meanwell, 1991).

Although the level of cGMP in the resting platelet is very low *in vitro*, stimulated

endothelial cells release NO, which may reach platelets flowing at the periphery of the

vascular lumen. Thus, inhibition of PDE3 and activation of PDE2 by cGMP in platelets

may be physiologically relevant processes, and in order to inhibit platelet responses

optimally, an inhibitor that targets PDE2 as well as PDE3 should be sought.


4.5.   Conclusion

The goal of this research was to gain an understanding of the role of PDE2 in

the regulation of platelet cyclic nucleotide levels and of the effects that PDE2 activity has

on platelet aggregation. Compounds that stimulate platelet adenylyl cyclase and guanylyl cyclase were used to provoke increases in cAMP and cGMP to determine the role of PDE2 in different conditions. A diagram of the factors involved in the regulation of platelet cyclic nucleotide concentrations is presented in Figure 20. Stimulators of soluble guanylyl cyclase, such as NP, lead to increases in cGMP, which has multiple targets within the platelet. cGMP is a substrate for PDE5 and for PDE2. cGMP also regulates the cAMP phosphodiesterases, in an opposing manner; cGMP inhibits PDE3 (and so increases cAMP levels), and higher concentrations of cGMP also stimulate PDE2 activity (tending to decrease cAMP). Thus, PDE2 and PDE3 may be thought of as mediators of cross-talk between cAMP and cGMP. The concentration of cAMP also determines which PDE is mainly responsible for its metabolism. At low levels of cAMP and in the absence of cGMP, PDE3 is the major cAMP phosphodiesterase activity, but at higher cAMP levels or in the presence of cGMP, the activity of PDE2 is more important. There are thus two cues which determine PDE2 activity: the concentrations of substrates and of regulatory cGMP.

The role of PDE2 may be to restrict the inhibition of platelet function. Accumulation of cAMP or cGMP, triggered by inhibitory agonists such as PGI_2 and NO, opposes platelet responses. Since cyclic nucleotides have only inhibitory effects on platelet aggregation, PDE2 is a unidirectional regulator which relieves inhibition by hydrolysing cyclic nucleotides, thereby restoring the reactivity of platelets.

Figure 20:   Regulation of platelet cyclic nucleotides by PDEs

The key factors involved in the regulation of platelet cyclic nucleotide levels are outlined

in this scheme. Enzymes are designated by the shaded symbols. Stimulation of enzymes

is denoted by $\oplus$, inhibition by $\ominus$. NP leads to the production of cGMP through guanylyl

cyclase, GC. cGMP is a substrate for both PDE5 and PDE2. PGI_2 stimulates adenylyl

cyclase, resulting in increases in platelet cAMP, which is a substrate for both PDE2 and

PDE3. cGMP exerts effects on cAMP levels through PDEs: it increases cAMP by

inhibiting PDE3, and decreases cAMP by stimulating PDE2.
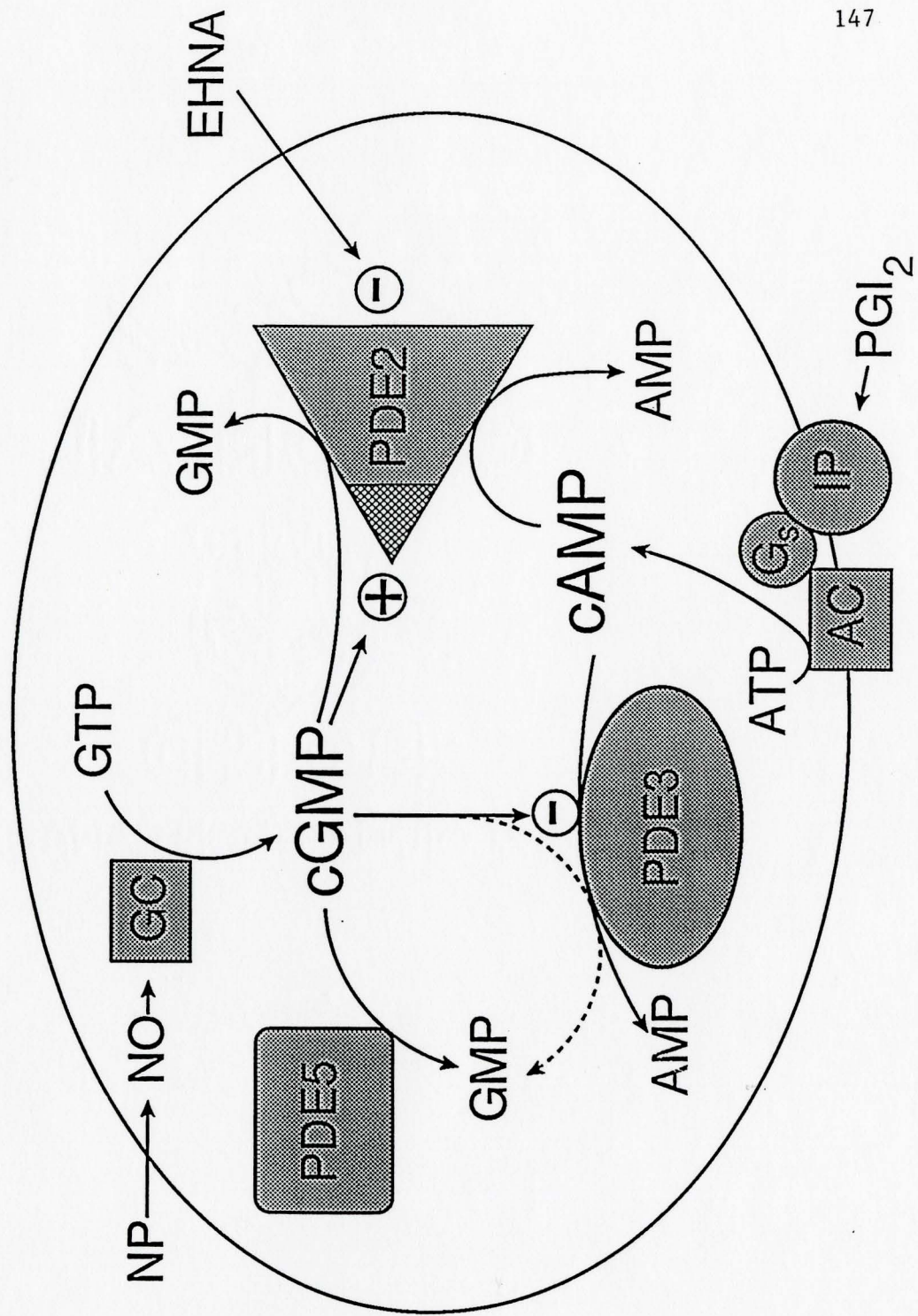
The present study greatly extends our current knowledge of the role of PDE2 in the platelet, but much remains to be investigated in this area. For example, the other PDEs present in platelets can be phosphorylated. Is PDE2 regulated by phosphorylation, or possibly by other post-translational modifications of the enzyme? Does PDE2 interact with any regulatory proteins? In light of this demonstration of the importance of PDE2 in platelet function, the regulation of this enzyme should be explored further.

This study is the first to use EHNA to investigate the role of PDE2 in the platelet. The utility of EHNA is demonstrated in this research, which suggests that this compound will make a significant contribution to our understanding of PDE2 in the platelet and in other systems. At the present time, no other selective inhibitor of PDE2 is known. The results suggest that a compound that combines inhibitory effects on PDE3 and PDE2 could have particularly potent effects on platelet function.

In summary, this research represents the first detailed study of the role of PDE2 in the human platelet. The results indicate that this enzyme plays a major role in the hydrolysis of platelet cyclic nucleotides. In fact, the importance of PDE2 may have been underestimated in previous studies. *In vivo*, platelets are constantly exposed to NO and PGI$_2$, both of which are released from endothelial cells and may increase platelet cyclic nucleotide levels. Thus, it is possible that PDE2 plays a highly significant role in the regulation of platelet function *in vivo*.

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