SIGNAL TRANSDUCTION IN BLOOD PLATELETS

SIGNAL TRANSDUCTION IN BLOOD PLATELETS: REGULATION OF ADENYLATE CYCLASE AND PHOSPHOLIPASE C

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ABSTRACT

Cells including platelets respond to many agonists through signal transduction mechanisms in the plasma membrane composed of specific receptors, guanine nucleotide-binding proteins (G proteins) and effector enzymes, which synthesize molecules that function as intracellular 'second messengers'. One of these enzymes, adenylate cyclase, which is subject to positive and negative regulation by different agonists, generates cyclic AMP (cAMP) that inhibits platelet responses. A second enzyme, phospholipase C, is stimulated by many agonists that induce platelet aggregation and secretion and generates diacylglycerol (DG) and inositol phosphates (including IP_2 and IP_3). DG activates protein kinase C (PKC), which has both stimulatory and inhibitory effects on platelet responses, whereas IP_3 mobilizes Ca^{2+} ions from intracellular stores, thereby stimulating many Ca^{2+}-dependent reactions involved in platelet activation. In this thesis, the effects of agonists that stimulate platelets, particularly platelet-activating factor (PAF), on platelet adenylate cyclase and phospholipase C activities have been studied with a view of defining some of the biochemical properties and interactions of these signal transduction pathways.

In platelet particulate fractions, PAF inhibited adenylate cyclase in a GTP-dependent manner. NaCl stimulated basal adenylate

cyclase activity and potentiated the inhibitory action of PAF in rabbit and human platelet particulate fractions, with the largest effects in the former species. An assay was developed that allowed measurement of both adenylate cyclase and phospholipase C activities under identical conditions in the same rabbit platelet particulate fraction. PAF and thrombin were potent stimulators of phospholipase C and inhibitors of adenylate cyclase, whereas epinephrine acted solely as an inhibitor of adenylate cyclase. Pertussis toxin ADP-ribosylated three proteins in the 38-42 kDa range in rabbit platelet particulate fractions. Although pertussis toxin diminished the effects of PAF on both enzymes, the inhibition of adenylate cyclase by epinephrine was much more susceptible to toxin treatment. These findings suggest that although pertussis toxin substrate(s) mediate in part both inhibition of adenylate cyclase and activation of phospholipase C by PAF, other G proteins may also be coupled to these enzymes.

In intact human platelets, PAF and low concentrations of thrombin failed to decrease cAMP formation caused by PGE_1 and IBMX under conditions in which epinephrine was effective. It was not possible to re-couple PAF receptors to adenylate cyclase in human platelets by addition of a low epinephrine concentration, by altering the intracellular or extracellular concentration of Na^+, by increasing the membrane fluidity or by depolymerizing platelet microtubules or microfilaments. To determine whether activation of

PKC by PAF or low thrombin blocked their ability to inhibit adenylate cyclase, a double labelling assay was developed to compare the effects of phorbol ester (PMA), PAF and thrombin on cAMP formation and protein phosphorylation in the same platelets. Although PMA was able to block the inhibition of cAMP formation by epinephrine, the activation of PKC by PAF or low thrombin was insufficient to account for their failure to inhibit cAMP formation. Moreover, high thrombin concentrations simultaneously activated PKC and inhibited cAMP formation. These results suggest that an unidentified cytosolic factor can block the inhibition of adenylate cyclase by PAF in intact human platelets.

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LIST OF ABBREVIATIONS

AA	arachidonic acid
ACD	acid citrate dextrose
ACS	aqueous counting scintillant
ADP	adenosine 5′-diphosphate
AMP	adenosine 5′-monophosphate
ANOVA	analysis of variance
ATP	adenosine 5′-triphosphate
AVP	[Arg^8]vasopressin
BSA	bovine serum albumin
C	catalytic unit of adenylate cyclase
$[Ca^{2+}_i]$	intracellular calcium ion concentration
$[Ca^{2+}_{free}]$	free calcium ion concentration
cAMP	adenosine cyclic 3′,5′-monophosphate
cGMP	guanosine cyclic 3′,5′-monophosphate
CP	creatine phosphate
CPK	creatine phosphokinase
c.p.m.	counts per minute
DFP	diisopropylfluorophosphate
DG	*sn*-1,2-diacylglycerol
diC_8	*sn*-1,2-dioctanoylglycerol
d.p.m.	disintegrations per minute
DTT	dithiothreitol
ED_{50}	concentration causing 50% of the maximum effect

EDTA	ethylenediamine tetraacetic acid
EGF	epidermal growth factor
EGTA	ethyleneglycol-bis-(β-aminoethyl ether) *N,N'*-tetraacetic acid
EPI	epinephrine
expts.	experiments
fMLP	*N*-formyl-methionyl-leucyl-phenylalanine
G protein	guanine nucleotide binding protein
$g_{av.}$	g force at the mean radius of the centrifuge tube
GDP	guanosine 5'-diphosphate
GDPβS	guanosine 5'-O-(2-thiodiphosphate)
Gpp[NH]p	guanyl-5'-yl $\beta\gamma$-imidodiphosphate
GTP	guanosine 5'-triphosphate
GTPγS	guanosine 5'-O-(3-thiotriphosphate)
Hepes	*N*-2-hydroxyethylpiperazine-*N'*-2-ethanesulphonic acid
HPLC	high performance liquid chromatography
5-HT	serotonin
H7	1-(5-isoquinolinesulphonyl)-2-methylpiperazine
IBMX	3-isobutyl-1-methylxanthine
IP_1	inositol monophosphate
IP_2	inositol bisphosphate
IP_3	inositol trisphosphate
Mops	3-(*N*-morpholino)propanesulphonic acid
Na^+_o	extracellular sodium ion
Na^+_i	intracellular sodium ion
NAD^+	nicotinamide adenine dinucleotide

$NADP^+$	nicotinamide adenine dinucleotide phosphate
NEM	*N*-ethylmaleimide
NMN	nicotinamide mononucleotide
PA	phosphatidic acid
PAF	platelet activating factor (usually synthetic 1-*O*-octadecyl-2-*O*-acetyl-*sn*-glyceryl-3-phosphocholine)
pCa	$-\log [Ca^{2+}_{free}]$
PKC	protein kinase C
4α-PDD	4α-phorbol 12,13-didecanoate
PGE_1	prostaglandin E_1
PGD_2	prostaglandin D_2
PGG_2	prostaglandin G_2
PGH_2	prostglandin H_2
PGI_2	prostacyclin
pH_i	intracellular pH
PI	phosphatidylinositol
PIP	phosphatidylinositol 4-phosphate
PIP_2	phosphatidylinositol 4,5-bisphosphate
Pipes	piperazine-*N*,*N*′-bis(2-ethanesulphonic acid)
PLA_2	phospholipase A_2
PMA	phorbol 12-myristate 13-acetate
PRP	platelet-rich plasma
SDS	sodium dodecyl sulphate
SEM	standard error of the mean
SED	standard error of the difference
SEQ	standard error of the quotient

t.l.c.	thin layer chromatography
TCA	trichloroacetic acid
Tris	Tris(hydroxymethyl)aminomethane
TxA_2	thromboxane A_2

Chapter 1

Introduction

1.1. *Platelet activation*

Platelets are discoid anucleate cells, 2-3 μm in diameter, that are formed in the bone marrow by fragmentation of the cytoplasm of megakaryocytes and are released into the blood stream to circulate for 6-10 days. Upon activation by physiological agonists, platelets can adhere to the subendothelium, change shape, aggregate and secrete their granule contents (Zucker and Nachmias, 1985; Steen and Holmsen, 1987). These platelet responses are central to the role of the platelet in haemostasis and thrombosis (Mustard *et al.*, 1981; Gerrard, 1988). At the site of injury, the blood vessel contracts and platelets adhere to the subendothelium by the binding of Von Willebrand factor and other adhesive plasma proteins to glycoproteins Ib and IIb/IIIa on the platelet surface (Steen and Holmsen, 1987). Transformation of the discoid platelet into a sphere with extending pseudopodia occurs within seconds of platelet activation (Born, 1970) and exposes glycoprotein IIa-IIIb, the receptor for fibrinogen, by a Ca^{2+}-independent process that requires ATP and a major rearrangement of the platelet cytoskeleton (Phillips *et al.*, 1988). For platelet aggregation to occur, activated platelets must be in close proximity in a medium containing Ca^{2+} and fibrinogen (Steen and Holmsen, 1987), so that cross-linking of platelets by fibrinogen can take place. Platelet aggregation can be reversible or irreversible, the latter requiring secretion of thrombospondin to stabilize the fibrinogen linkages between platelets. Platelets contain dense, α and acid hydrolase-

containing granules which release their contents to varying degrees depending on the platelet stimulus. The platelet granules contain the following components: ADP, ATP and 5-HT in the dense granules; fibrinogen, other clotting factors, thrombospondin and growth factors in the α granules; β-glucuronidase and other enzymes in the lysosomes. Platelet secretion occurs by a Ca^{2+}-promoted mechanism that requires metabolic ATP and acts to amplify ongoing platelet activation and to recruit circulating platelets by releasing aggregating agents (ADP, serotonin) and adhesive proteins (fibrinogen, thrombospondin) into the surrounding medium. A hemostatic plug or platelet thrombus initially consists of an aggregate of platelets at the injury site. This develops from the initial platelet activation by subendothelial collagen fibres through positive feedback effects exerted by released granule constituents and prostaglandin endoperoxides and TxA_2. The latter are synthesized from AA released from the membrane phospholipids (Steen and Holmsen, 1987). Concurrent activation of the intrinsic and extrinsic pathways of the coagulation system ultimately reinforces the platelet aggregate, through the formation of thrombin from the inactive plasma protein, prothrombin. Thrombin causes further platelet activation and the formation of fibrin from fibrinogen. A mature haemostatic plug thus, forms at the site of injury by the addition of fibrin polymers to the platelet aggregate.

The agonists which cause platelet activation *in vitro*

include thrombin, collagen fibres, TxA_2, ADP, AVP, PAF, 5-HT and epinephrine. Of these, only thrombin, collagen fibres, TxA_2 and ADP are thought to play major roles in the pathophysiology of human platelets (Zucker and Nachmias, 1985; Haslam, 1987). As PAF is a more potent activator of rabbit than human platelets and does not depend on released ADP or the production of prostaglandin endoperoxides and TxA_2 to activate rabbit platelets, it is more likely to be of significance in this species (see Section 1.1.6).

1.2. *Signal transduction in blood platelets*

Platelets respond to a wide variety of physiological compounds (hormones, autacoids, neurotransmitters, coagulation factors and vascular proteins) which can either activate or inhibit platelet function (reviewed by MacIntyre *et al.*, 1986). Since most extracellular mediators cannot pass through the plasma membrane and platelets are not activated by changes in membrane potential (Rink, 1986), signals are conveyed from the external milieu to the interior of the cell by specific signal transduction mechanisms, composed of receptor (Lefkowitz and Caron, 1988), transducer (Gilman, 1987; Spiegel, 1987) and effector molecules (Jakobs *et al.*, 1981; Litosch and Fain, 1986; Casperson and Bourne, 1987). The presence of a number of different types of receptors for these agonists on the platelet membrane surface combined with the ease of preparing a homogeneous population of these cells makes the platelet an ideal

model system for studying signal transduction. The rapidly formed and transient molecules produced from signal transfer across cell membranes activate intracellular targets to induce cellular responses and thus have been called second messengers. In the platelet, Ca^{2+} and cAMP were originally thought to be the sole second messengers governing activation and inhibition of platelet function, respectively. However, it is now clear that the products of phosphoinositide metabolism, DG (Nishizuka, 1984) and IP_3 (Berridge and Irvine, 1984) have important second messenger functions in platelet activation. In addition, cGMP (Haslam *et al.*, 1978a) and increases in pH_i (Sweatt *et al.*, 1985, 1986a,b; Siffert *et al.*, 1984, 1987; Siffert and Akkerman, 1987, 1988) are also thought to act as second messengers controlling platelet reactivity; however, their function is less completely understood.

In the platelet, two main signal transduction pathways are physiologically relevant, PI breakdown/Ca^{2+} mobilization and the production of cAMP. The former is central to platelet activation and the actions of the second messengers produced, IP_3/Ca^{2+} and DG, are mediated at least in part by Ca^{2+}/calmodulin-dependent protein kinases and PKC, respectively. In contrast, elevated levels of cAMP inhibit platelet function by activating cAMP-dependent protein kinases. Thus, the action of the two main second messengers in platelets are opposing (reviewed by Kikkawa and Nishizuka, 1986). Bidirectional control of signal transduction also occurs in

lymphocytes, mast cells and neutrophils (reviewed in Kikkawa and Nishizuka, 1986). However, monodirectional control of signal transduction in which the PI breakdown/Ca^{2+} mobilization pathway reinforces the production of cAMP is common to many other mammalian cells (reviewed in Kikkawa and Nishizuka, 1986).

PKC and cAMP-dependent protein kinases may also function to regulate the synthesis and/or metabolism of the second messenger molecules themselves (reviewed by Kikkawa and Nishizuka, 1986). For example, hormone-stimulated PI breakdown and Ca^{+} mobilization are subject to negative feedback by PKC and cAMP-dependent protein kinase. PKC may also regulate the production of cAMP in many cells.

1.3. *Adenylate cyclase*

The role of cAMP as an intracellular mediator of a wide spectrum of physiological responses caused by hormones and neurotransmitters was uncovered by Sutherland and co-workers over a 12 year period (reviewed by Sutherland, 1970). Originally, a dialysable compound residing in the cytosol but produced by the particulate fraction was found to be responsible for the activation of phosphorylase by glucagon or epinephrine in liver homogenates (Rall *et al.*, 1956). The identity of the soluble compound was determined to be cAMP, which was synthesized from MgATP by adenylate cyclase (Rall and Sutherland, 1962) and degraded to 5'-AMP by

cAMP-dependent phosphodiesterase (Butcher and Sutherland, 1962).

$$ATP \xrightarrow{\text{adenylate cyclase}} PP_i + cAMP \xrightarrow{\text{cAMP phosphodiesterase}} 5'\text{-}AMP$$

Following the discovery of the role of cAMP in hormone action in many different types of cells and tissues, Sutherland (1970) characterized cAMP as a "second messenger" that mediates the intracellular effects of hormones ("first messengers") acting on the outside of the cell.

Initially, adenylate cyclase was thought to be a single molecule that was regulated allosterically by the agonist (Robison *et al.*, 1967; Stellwagen and Baker, 1976), as is the nicotinic acetylcholine receptor, which contains the recognition, transduction and effector functions within a single multimeric protein (Changeux *et al.*, 1984). However, it was soon demonstrated that the adenylate cyclase system was composed of three separable components present in the plasma membrane, the receptor, the catalytic unit and a GTP-binding protein (G protein) (reviewed by Ross and Gilman, 1980). This multicomponent enzyme system amplifies the initial hormonal signal more than a 1000-fold upon activation of appropriate membrane receptors and is found in most eukaryotic cells. Adenylate cyclase can be stimulated or inhibited by specific agonists and, in the platelet, receptors for both pathways exist. The physiological effect of elevated levels of cAMP is mediated by the phosphorylation

of cellular proteins by specific protein kinases that the cyclic nucleotide activates (Krebs and Beavo, 1979). In the platelet, increases in cAMP block platelet aggregation via the cAMP-dependent phosphorylation of specific protein kinase substrates (Haslam *et al.*, 1978a). However, decreases in cAMP in the platelet do not mediate platelet aggregation, as originally proposed (Salzman and Levine, 1971), and are thought only to reduce the impact of compounds such as PGE_1 and PGI_2 that inhibit platelet function by activating adenylate cyclase (Haslam and Taylor, 1971; Haslam *et al.*, 1978a,b). Extensive research with many different types of cells has now revealed the complexity of the regulatory apparatus responsible for this important signal transduction system (reviewed by Limbird, 1981; Codina *et al.*, 1984; Gilman, 1987).

1.3.1. *Stimulation of adenylate cyclase*

Role of guanine nucleotides. Activation of adenylate cyclase by agonists was first shown to be dependent on guanine nucleotides by Rodbell *et al.* (1971a,b). Guanine nucleotides both enhanced the stimulation of adenylate cyclase by glucagon (Rodbell *et al.*, 1971a) and decreased the affinity of the receptor for this agonist in rat liver plasma membranes (Rodbell *et al.*, 1971b). These effects of guanine nucleotides did not involve binding of GTP to the glucagon receptor (Rodbell *et al.*, 1971b) and were subsequently shown to be common to the activation of

adenylate cyclase by all physiological agonists in all cells and tissues examined (reviewed by Limbird, 1981). They were specific for guanine nucleotides and metabolically stable GTP analogs such as Gpp(NH)p or GTPγS, had higher potencies than GTP itself. The concentration of GTP causing half-maximal effects ranged from 10^{-8}-10^{-7} M (Limbird, 1981) and the action of GTP on hormone-stimulated adenylate cyclase was competively inhibited by GDP (Salomon *et al.*, 1975) or GDPβS (Eckstein *et al.*, 1979). Cassel and Selinger (1976) reported both increased GTPase activity and [^{3}H]GDP release in catecholamine-stimulated turkey erythrocyte membranes (1978). Stable GTP analogues caused persistent activation of adenylate cyclase activity (Schramm and Rodbell, 1975) and the lag period before this effect was observed was reduced by the presence of hormone (Londos *et al.*, 1974). Potentiation of hormone and GTP-induced stimulation of adenylate cyclase by cholera toxin was found to be due to the inhibition of GTPase activity (Cassel and Selinger, 1977). Together, these findings pointed to the importance of hormone-facilitated GDP/GTP exchange and hydrolysis of GTP by GTPase in the activation and deactivation of hormone-stimulated adenylate cyclase, respectively.

Identification of the coupling protein in hormone--stimulated adenylate cyclase. The above actions of guanine nucleotides on hormone-stimulated adenylate cyclase are mediated by a GTP-binding membrane protein (G_s) that is distinct from the

receptor and catalytic subunit of adenylate cyclase (reviewed by Ross and Gilman, 1980). Studies demonstrating photoaffinity labelling of a 42 kDa GTP-binding protein in pigeon erthrocyte membranes (Pfeuffer, 1977), the restoration of Gpp(NH)p and NaF-stimulated adenylate cyclase activity on addition of partially purified 42 kDa protein to cyc^- cells (Pfeuffer, 1977) and ADP-ribosylation of a 42 kDa protein by cholera toxin (Cassel and Pfeuffer, 1978), all suggested that this GTP-binding protein was responsible for the guanine nucleotide sensitivity of hormone-stimulated adenylate cyclase. It became possible to purify homogeneous G_s from rabbit liver (Northup *et al.*, 1980; Sternweis *et al.*, 1981) and from turkey and human erythrocyte plasma membranes (Hanski *et al.*, 1981; Hanski and Gilman, 1982) by column chromatography, using a reconstitution assay with the cyc^- mutant of S49 lymphoma cells. The latter possesses functional β-adrenergic receptors and catalytic unit but lacks G_s (reviewed by Ross and Gilman, 1980).

Characterization of G_s. G_s can modify the affinity of the receptor for the hormone and the activity of the catalytic subunit (reviewed by Limbird, 1981). It is also one of the proteins through which Mg^{2+} regulates the activity of adenylate cyclase (reviewed in Gilman, 1987). Furthermore, incorporation of purified β-adrenergic receptor, G_s and catalytic unit into phospholipid vesicles reconstitutes hormone-sensitive adenylate cyclase suggesting that these three components are necessary and sufficient for

GTP-dependent hormone-stimulated cAMP formation (May *et al.*, 1985). G_s is a heterotrimer composed of α (52 or 45 kDa), β (35-36 kDa) and γ (8 kDa) subunits with a 1:1:1 stoichiometry (reviewed in Gilman, 1987). Both forms (Northup *et al.*, 1980) or only one form (Codina *et al.*, 1984) of the α_s subunit has been observed in the same cell type and it appears that different cells contain different proportions of these proteins (Gilman, 1987). cDNA studies have demonstrated the existence of four distinct species of α_s (Robishaw *et al.*, 1986; Bray *et al.*, 1986), two of which have been shown to reconstitute stimulation of adenylate cyclase activity by hormone, GTPγS and NaF in cyc$^-$ membranes (Graziano *et al.*, 1987). The isolation, sequencing and expression of two G_s-specific cDNA clones that code for the 52 and 45 kDa proteins suggest that the two distinct mRNAs that are generated, arise from a single gene by alternative splicing (Robishaw *et al.*, 1986). The α_s subunit contains GTPase activity, the GTP binding site and the arginine residue that is ADP-ribosylated by cholera toxin (reviewed in Graziano and Gilman, 1987). In the absence of $\beta\gamma$, activated α_s can stimulate adenylate cyclase in cyc$^-$ cells and directly activate the catalytic unit independently of any other components of this enzyme system (Northup, 1983a). The $\beta\gamma$ subunits may oppose hormone-induced activation of adenylate cyclase (Northup, 1983b) and may also function to secure G_s to the plasma membrane (Sternweis, 1986).

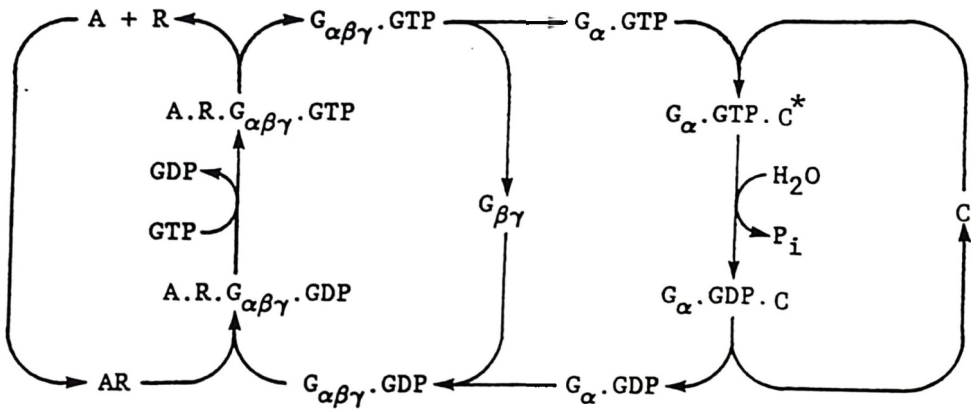
Mechanism of hormone-induced activation of adenylate cyclase. Activation of adenylate cyclase by hormones and other agonists involves the sequential tranfer of information from the receptor to G_s to the catalytic unit of adenylate cyclase (see Fig. 1.1). Agonist occupation of the receptor causes receptor-G_s coupling. In this conformation, the receptor has a high affinity for the agonist and the agonist-receptor complex accelerates the otherwise slow GDP/GTP exchange on α_s by inducing both the release of GDP and binding of GTP. The rate-limiting step in the activation of adenylate cyclase is the Mg^{2+}-dependent binding of GTP to α_s. Formation of the agonist-receptor complex decreases the concentration of Mg^{2+} required for this step. Binding of GTP to α_s in the presence of Mg^{2+} has two major effects. It causes the dissociation of the receptor-G_s complex, which results in a lower affinity of the receptor for agonist, and probably also induces the reversible dissociation of G_s into α_s and $\beta\gamma$, though this has only been directly demonstrated with GTPγS (Northup *et al.*, 1983a). α_sGTP now activates the catalytic unit of adenylate cyclase which converts intracellular ATP into cAMP and pyrophosphate. Activation of adenylate cyclase is probably terminated by the hydrolysis of GTP to GDP by the low K_m GTPase activity of α_s. The basal activity of this enzyme is very low and is increased substantially by the GDP/GTP exchange caused by the agonist-receptor complex. α_sGDP then reassociates with $\beta\gamma$. Free $\beta\gamma$ accelerates the rate of deactivation of α_s (Northup

et al., 1983b) and slows the dissociation and activation of G_s by Mg^{2+} and GTPγS (Northup *et al.*, 1982). This mechanism can amplify the hormonal signal more than a thousand-fold, because of the catalytic action of the receptor (interacts with ~ 10 G_s molecules before the agonist dissociates) and the fairly long half-life of activated α_s (Gilman, 1987).

The above model for hormonal stimulation of adenylate cyclase (Fig. 1.1) is based on the cycle of GDP/GTP exchange and GTP hydrolysis discovered by Cassel and Selinger (1977; 1978) and of G_s subunit dissociation and reassociation reported by Gilman and co-workers (1987). Although the model can account for many of the findings obtained upon investigation of the mechanistic aspects of hormone-stimulated adenylate cyclase from a large number of laboratories, there is some question of whether subunit dissociation is physiological or even required for G_s activation in the membrane environment (reviewed in Levitzki, 1987a). Also, the model implies a independent mobility of the three components of the adenylate cyclase system, which is not consistent with experiments demonstrating first order kinetics for hormone and guanine nucleotide-induced activation of adenylate cyclase in both artifical and physiological systems (Levitzki, 1987a).

Fig. 1.1. Mechanism of activation of adenylate cyclase by agonists

Agonist occupation of the receptor causes receptor-G_s coupling. In this conformation, the receptor has a high affinity for agonist and the agonist-receptor complex accelerates the otherwise slow GDP/GTP exchange on α_s by inducing both the release of GDP and binding of GTP. Binding of GTP to α_s causes dissociation of the receptor-G_s complex, which results in a lower affinity of the receptor for agonist and probably also induces the reversible dissociation of G_s into α_s and $\beta\gamma$ subunits. α_sGTP activates the catalytic unit of adenylate cyclase which converts intracellular ATP into cAMP and pyrophosphate. Activation of adenylate cyclase is probably terminated by the hydrolysis of GTP to GDP by the low K_m GTPase activity of α_s. α_sGDP then reassociates with $\beta\gamma$. Abbreviations: A, agonist; R, receptor; AR, agonist-receptor complex; $G_{\alpha\beta\gamma}$, heterotrimeric form of G_s; A.R.$G_{\alpha\beta\gamma}$, agonist-receptor-G_s complex; C, catalytic unit of adenylate cyclase: C^*, activated catalytic unit of adenylate cyclase. This figure has been taken from Gilman, 1987.



1.3.2. *Inhibition of adenylate cyclase*

Although epinephrine was reported to decrease cAMP accumulation caused by PGE_1 in intact platelets as early as 1969 (Robison *et al.*, 1969), very little was discovered about the inhibition of adenylate cyclase before 1976 because of difficulties in detecting decreases in enzyme activity in membrane preparations. It then became apparent that receptor-mediated inhibition of adenylate cyclase was caused by a variety of agonists in membranes prepared from many different cells and tissues (reviewed by Jakobs *et al.*, 1981). The bimodal regulation of adenylate cyclase and the characterization of the inhibition of this enzyme was initially investigated in the human platelet (Jakobs *et al.*, 1981) and the rat fat cell (Cooper, 1982), which contain receptors that induce either stimulation or inhibition of adenylate cyclase.

Properties of the hormonal inhibition of adenylate cyclase. Typically, hormonal inhibition of adenylate cyclase occurred without delay, showed good correlation with receptor binding and was immediately reversed by specific receptor antagonists, suggesting that it was attributable to a receptor-mediated inhibition of the enzyme (Jakobs *et al.*, 1981). Although the extent of inhibition of adenylate cyclase varied with the agonist concentration, the percentage inhibition was usually greatest with respect to basal adenylate cyclase activity and was decreased in the

presence of a stimulatory agonist (Jakobs *et al.*, 1981; Cooper, 1982). Since the inhibition of adenylate cyclase by different agonists acting on distinct receptors was non-additive, a common pool of adenylate cyclase was thought to couple to these receptors (Jakobs *et al.*, 1981; Cooper, 1982). In human platelet membranes, epinephrine (Jakobs *et al.*, 1976), ADP (Cooper and Rodbell, 1979), PAF (Haslam and Vanderwel, 1982), AVP (Vanderwel *et al.*, 1983) and thrombin (Aktories and Jakobs, 1984) inhibit adenylate cyclase activity in a guanine nucleotide-dependent manner.

Investigation of the regulatory aspects of hormonal inhibition of adenylate cyclase revealed a number of features common to the inhibitory and stimulatory pathways. Similar to the activation of adenylate cyclase, hormonal inhibition of adenylate cyclase was regulated by Mg^{2+}, was dependent on GTP and was inhibited by GDP (reviewed by Gilman, 1987). However, the concentration of GTP required for half-maximal inhibition of adenylate cyclase was 5-10 fold greater (Jakobs *et al.*, 1981; Cooper, 1982) and the optimal [Mg^{2+}] for hormonal inhibition of adenylate cyclase was much lower (Gilman, 1987) than were the concentrations required for stimulation of the enzyme by agonists. Like stimulatory agonists, inhibitory agonists activated a low K_m GTPase in direct proportion to their ability to inhibit adenylate cyclase but in the human platelet, epinephrine stimulated GTP hydrolysis to a much greater extent than did PGE_1 (Aktories and

Jakobs, 1981). Stable analogs of GTP persistently activated or inhibited adenylate cyclase under appropriate conditions (Jakobs and Aktories, 1983), by virtue of their resistance to hydrolysis by the GTPase activities which acted as turn-off mechanisms for both reactions. Both stimulatory agonists (Londos *et al.*, 1974) and inhibitory agonists (Jakobs and Aktories, 1983) shortened the lag period before development of stimulation or inhibition of adenylate cyclase caused by stable GTP analogs. Guanine nucleotides also reduced the agonist affinity of α_2-adrenoceptors coupled to the inhibition of adenylate cyclase in rabbit (Michel *et al.*, 1980) and human (Tsai and Lefkowitz, 1979a; Hoffman *et al.*, 1982) platelet membranes. As with the stimulation of adenylate cyclase, shifts from high to low affinity states of the receptor were thought to reflect the guanine nucleotide-induced dissociation of the hormone-receptor complex from a G protein (Hoffman *et al.*, 1982).

Differences were also observed between hormonal stimulation and hormonal inhibition of adenylate cyclase. In addition to the requirements for higher concentrations of GTP and lower amounts of Mg^{2+}, monovalent cations were also required for optimal hormonal inhibition of adenylate cyclase activity. With a characteristic order of potency of $Na^+ \geq Li^+ > K^+ > choline^+$, monovalent cations enhanced agonist-induced inhibition of adenylate cyclase activity in adipocytes (Aktories *et al.*, 1981) and neuroblastoma x glioma cells (Blume *et al.*, 1979). Sodium ions were also

reported to decrease α_2-adrenoceptor affinity for epinephrine in rabbit (Michel *et al.*, 1980) and human (Limbird *et al.*, 1982) platelet membranes. In contrast to the facilitatory action of monovalent cations on the inhibitory pathway of adenylate cyclase, these ions have also been reported to attenuate hormonal stimulation of adenylate cyclase in adipocyte (Aktories *et al.*, 1981) and human platelet membranes (Steer and Wood, 1981).

The other major feature distinguishing the stimulatory and inhibitory pathways affecting adenylate cyclase activity is their differential susceptibility to agents which modify or perturb receptor-effector coupling. Cholera toxin persistently activates adenylate cyclase by inhibiting the GTPase activity (Cassel and Selinger, 1977). However, cholera toxin treatment of human platelet membranes does not affect the inhibition of PGE_1-stimulated adenylate cyclase by epinephrine or the stimulation of GTPase activity by epinephrine in the same preparations that it modifies the action of PGE_1 (Aktories *et al.*, 1982a). Furthermore, cholera toxin treatment of human platelet membranes does not modify the affinity of the α_2-adrenergic receptor for agonists and the cholera toxin substrate does not migrate with the solubilized α_2-adrenergic agonist-receptor complex upon sucrose gradient centrifugation (Smith and Limbird, 1982). Treatment of human platelet membranes with NEM, trypsin or low concentrations of Mn^{2+} abolishes inhibition of adenylate cyclase by agonists and GTP, while

leaving the stimulatory pathway intact (Jakobs *et al.*, 1982; Aktories and Jakobs, 1984; Stiles and Lefkowitz, 1982). These observations support the hypothesis put forward by Rodbell (1980) that receptors coupled to the stimulatory and inhibitory pathways of adenylate cyclase are linked to distinct G proteins.

Characterization of G_i. Although studies of the dual regulation of adenylate cyclase supported similar but distinct pathways for stimulation and inhibition of the enzyme by agonists, the G protein thought to mediate GTP-dependent inhibition of adenylate cyclase by agonists (G_i) was identified in an independent investigation. Ui and co-workers discovered that the inhibition of adenylate cyclase by epinephrine was attenuated by pertussis toxin and that this effect correlated with [^{32}P]ADP-ribosylation of a 41 kDa protein distinct from the cholera toxin substrate (reviewed in Ui, 1984). Blockade by pertussis toxin of the inhibition of adenylate cyclase by GTP (Hildebrandt *et al.*, 1983) and somatostatin (Aktories *et al.*, 1983a) in cyc$^-$ cells which lack G_s, provided further evidence that a novel G protein mediates the inhibitory pathway. Pertussis toxin, purified from the supernatant of cultures of *Bordetella pertussis*, has a molecular mass of 77 kDa (Yajima *et al.*, 1978) and is composed of a binding subunit (a pentamer, S_2-S_5) and the active S_1 subunit (Tamura *et al.*, 1982). In its native form, pertussis toxin exists as an A-B toxin, in that the A (S_1) subunit is transfered into cells upon binding of

the B (S_2-S_5) component to specific membrane receptors on the outside of the plasma membrane (Tamura *et al.*, 1983). The S_1 subunit contains an ADP-ribosyltransferase activity which upon separation from the B subunit and reduction of intrapeptide disulphide bonds (achieved with DTT *in vitro*) covalently modifies the 41 kDa protein by incorporation of the ADP-ribose moiety of NAD^+ (Katada *et al.*, 1983).

$$NAD^+ + \text{protein} \xrightarrow{\text{ADP-ribosyltransferase}} \text{protein-ADP-ribose} + \text{nicotinamide}$$

Pertussis toxin proved to be a valuable tool in the purification and characterization of the G protein thought to mediate the inhibition of adenylate cyclase. G_i, purified by a modification of the method used for G_s, was first isolated from rabbit liver (Bokoch *et al.*, 1983) and human erythrocyte plasma membranes (Codina *et al.*, 1983). Like G_s and G_T, G_i is a heterotrimer composed of α, β and γ subunits (Bokoch *et al.*, 1984; Hildebrandt *et al.*, 1984). The α subunit of G_i contains the guanine nucleotide-binding and ADP-ribosylation sites (Bokoch *et al.*, 1983) and GTPase activity (reviewed by Casey and Gilman, 1988). Examination of the amino acid composition and peptide maps of G_s, G_T and G_i revealed that these G proteins are not only functionally but also structurally similar (Manning and Gilman, 1983). Amino acid sequencing of cDNAs of G

proteins showed that a G_i-related protein, G_T and G_o are 65% homologous with each other and that G_s is 45% homologous with the other three G proteins (reviewed by Casperon and Bourne, 1987). These structural differences combined with the inability of G_s to interact with receptors that do not normally stimulate adenylate cyclase has suggested that G_s is the most dissimilar of these three G proteins (reviewed by Casperon and Bourne, 1987).

Comparison of the properties of G_i to those of other pertussis toxin substrates. In bovine brain, three pertussis toxin subtrates with apparent molecular masses of 41 kDa, 40 kDa and 39 kDa were identified but it was initially unclear whether or not the two lower molecular mass species were proteolytic fragments of G_i (Neer *et al.*, 1984). The 41 kDa and the 39 kDa proteins have been purified from bovine brain (Sternweis and Robishaw, 1984; Neer *et al.*, 1984) and rat brain (Katada *et al.*, 1986) and recently, the 40 kDa protein has been isolated from bovine and porcine brain (Katada *et al.*, 1987a). The 39 kDa polypeptide is the most abundant pertussis toxin substrate in mamalian brain constituting ~1% of the membrane protein. Lesser amounts of the 41 kDa protein are present and the 40 kDa protein accounts for the smallest fraction (Katada *et al.*, 1987a; Itoh *et al.*, 1988). The 39 kDa protein, referred to as G_o because of the failure to find a definitive function, is very similar to G_i in many respects (reviewed in Rouot *et al.*, 1987). Like G_s, G_i and G_T, G_o

is a heterotrimer that dissociates into α_{39} and $\beta\gamma$ subunits upon exposure to Mg^{2+} and stable analogs of GTP in detergent solution (Huff *et al.*, 1985). The α subunit of G_o possesses GTPase activity, a high-affinity guanine nucleotide-binding site and a cysteine residue near the carboxy terminus that can be ADP-ribosylated by pertussis toxin or alkylated by NEM (reviewed by Rouot *et al.*, 1987; Ui and Katada, 1987).

The α-subunits of both G_i and G_o contain an arginine residue at the position ADP-ribosylated by cholera toxin, though neither of these polypeptides are substrates for this toxin (Angus *et al.*, 1986; Itoh *et al.*, 1986). However, differences in peptide maps, GTPase activity, affinity for $\beta\gamma$ subunits, rate of GTPγS binding, affinity for Mg^{2+} and sensitivity to thermal denaturation indicated that G_i and G_o are distinct G proteins (reviewed in Rouot *et al.*, 1987).

The 40 kDa pertussis toxin substrate first identified in bovine brain (Neer *et al.*, 1984) and subsequently purified from porcine (Katada *et al.*, 1987a; Itoh *et al.*, 1988), bovine (Mumby *et al.*, 1988) and rat (Itoh *et al.*, 1988) brain is also a heterotrimer composed of $\alpha_{40}\beta\gamma$. The α_{40} subunit containing the guanine nucleotide-binding and ADP-ribosylation sites does not cross-react with some antibodies to α_{41} or α_{39} and yields a different peptide map from

α_{41} and α_{39} upon proteolysis of the [^{32}P]ADP-ribosylated α subunits (Katada *et al.*, 1987a). In addition, α_{41} did not cross-react to antiserum specific to α_{40} and α_{40} did not cross-react to antiserum specific to α_{39} (Mumby *et al.*, 1988). The lower molecular mass pertussis toxin substrates thus appear to be distinct proteins and not proteolytic fragments of the the 41 kDa protein.

At the time of the discovery of G_o in brain, only one pertussis toxin substrate of ~41 kDa was observed in other tissues and cells examined. However, improvements to the separation of these proteins combined with immunological and cDNA sequencing studies have indicated the existence of four distinct pertussis toxin substrates in addition to the retinal G_T proteins. Three cDNA clones coding for the 41 kDa, 40 kDa and an initially unidentified pertussis toxin substrate have been referred to as $G_{i\alpha1}$, $G_{i\alpha2}$ and $G_{i\alpha3}$, respectively, because their translated amino acid sequences are 85-94% homologous with each other (Jones and Reed, 1987; Itoh *et al.*, 1988). It remains unclear whether one or all of these can be coupled to the inhibition of adenylate cyclase since the earlier preparations of 'G_i' probably contained mixtures of these components. The fourth cDNA clone codes for the 39 kDa protein, G_o, which is the predominant pertussis toxin substrate in brain (Itoh *et al.*, 1988). The four distinct mRNAs are differentially expressed in a tissue-dependent manner. Since Southern analysis has

demonstrated that these four cDNA clones hybridize with distinct genomic DNA fragments, it is likely that they arise from four distinct genes and not from alternative splicing of the primary transcript of a single gene (Jones and Reed, 1987).

G_s, G_i, and G_T contain $\beta\gamma$ subunits that are functionally interchangeable and contain similar amino acid sequences and patterns of proteolytic fragments (reviewed in Gilman, 1987). However, development of antisera to β and γ, isolation of cDNA clones of β and γ subunits and their sequencing have determined that these subunits are more heterogeneous than previously thought (reviewed in Gilman, 1987; Neer and Clapham, 1988). In non-retinal cells, the β subunit of G_s, G_i and G_o is a doublet consisting of 35 kDa and 36 kDa proteins that separate from the α subunit upon activation but remain with the γ subunit under nondenaturing conditions. In the retina, G_T contains a 36 kDa β subunit with an amino acid sequence that is the same as that found in the 36 kDa subunit of nonretinal cells and a γ subunit immunologically distinct from that found in nonretinal cells. Immunological and cDNA sequencing studies suggest that the 35 kDa and 36 kDa are distinct proteins that are products of separate genes (Amatruda *et al.*, 1988). There is also evidence for heterogeneity of γ subunits in both retinal and non-retinal cells.

The pertussis toxin substrates are the heterotrimeric form ($\alpha\beta\gamma$) of G_i, G_o (Neer, 1984), G_T (Van Dop *et al.*, 1984) and the 40 kDa G protein (Katada *et al.*, 1987a) and not the activated free α subunits. Although the $\beta\gamma$ subunits are critical to the ADP-ribosylation of these G proteins by pertussis toxin, it still remains unclear how pertussis toxin blocks the inhibition of adenylate cyclase by agonists and guanine nucleotides. Since the heterotrimer is the substrate for pertussis toxin and fewer $\beta\gamma$ subunits were released by epinephrine and GTPγS in extracts of pertussis toxin-treated human platelet membranes, Katada *et al.* (1984a) proposed that ADP-ribosylation of the α subunit prevents the activation of the G protein by blocking its dissociation. However, stable analogues of GTP inhibit adenylate cyclase in some pertussis toxin-treated membranes (Cote *et al.*, 1984; Hildebrandt *et al.*, 1983; Jakobs *et al.*, 1984a) and hydrodynamic studies by Huff and Neer (1986) have demonstrated that the α and $\beta\gamma$ subunits of ADP-ribosylated G_i and G_o still dissociate upon exposure to GTPγS. ADP-ribosylation of the 41 kDa protein has no effect on GTPγS binding, GTPase activity or on the ability of α_i subunit to inhibit the catalytic unit of adenylate cyclase (Katada *et al.*, 1986). Pertussis toxin treatment does, however, prevent the guanine nucleotide-induced shift in receptor affinity for agonists, suggesting that ADP-ribosylation of α subunits blocks hormone-receptor coupling to the G protein (Cote *et al.*, 1984). This site of action of pertussis toxin has

been challenged by Jakobs *et al.* (1984a). These investigators found that pertussis toxin prolonged the lag period of activation of adenylate cyclase by GTPγS and suggested that guanine nucleotide activation of G_i and not inhibitory receptor-G_i coupling was perturbed in pertussis toxin-treated cells.

Proposed mechanisms of hormonal inhibition of adenylate cyclase. Hormone-induced GTP-dependent inhibition of adenylate cyclase can be observed upon reconstitution of purified (but probably heterogeneous) G_i into pertussis toxin-treated human platelet membranes suggesting that this G protein mediates the inhibitory regulation of the enzyme (Smigel, 1984). To determine the mechanism by which G_i inhibits adenylate cyclase, the effects of purified α_i and $\beta\gamma$ subunits on the adenylate cyclase activities of human platelet membranes (Katada *et al.*, 1984a) and wild type and cyc$^-$ S49 lymphoma cell membranes (Katada *et al.*, 1984b) were examined, as later were their effects on the partially purified catalytic unit in solution (Katada et al., 1986; 1987b). Although the mechanism by which G_i inhibits adenylate cyclase is still very controversial, these studies have suggested at least four modes of inhibition of the enzyme by the components of G_i, all of which are dependent on dissociation of the α and $\beta\gamma$ subunits upon activation; (1) free $\beta\gamma$ subunit from G_i associates with activated α_s to cause its deactivation, (2) free α_i inhibits the catalytic unit of adenylate cyclase directly, (3) free

$\beta\gamma$ inhibits the catalytic unit directly and (4) free $\beta\gamma$ inhibits the catalytic unit via binding to calmodulin.

The majority of the inhibition of adenylate cyclase in human platelets and S49 lymphoma cell membranes has been attributed to the action of $\beta\gamma$ (Katada *et al.*, 1984a,b). The inhibition of forskolin-stimulated adenylate cyclase by the 35 kDa subunit (shown later to contain both β and γ) was reversed by addition of the 41 kDa α-subunit and was non-additive with the effect of epinephrine plus GTPγS, which correlated with the increase in the amount of free 35 kDa subunit. The unliganded 41 kDa subunit did not inhibit the enzyme, but instead increased basal and hormone-stimulated adenylate cyclase activity. High concentrations of the 41 kDa subunit containing bound GTPγS were inhibitory, but to a lesser extent than the $\beta\gamma$ subunit. These findings formed the basis of the hypothesis that inhibition of adenylate cyclase is mediated by the suppression of activated α_s, through the association of $\beta\gamma$ from G_i with α_s (Katada *et al.*, 1984a,b). Since all cells examined to date contain much higher concentrations of G_i than G_s (~10-fold; Spiegel, 1987), a large reservoir of $\beta\gamma$ subunits can be made available by inhibitory agonists for attenuation of α_s activity. In cyc$^-$ membranes, inhibition of adenylate cyclase by somatostatin and GTP can be demonstrated even though these cells do not contain functional G_s, suggesting that other mechanisms for attenuating enzyme activity must

exist besides the deactivation of α_s by $\beta\gamma$ subunits (Katada *et al.*, 1984b). Incubation of cyc$^-$ membranes with the resolved subunits of G_i demonstrated that GTPγS-bound α_i inhibits and the $\beta\gamma$ subunit stimulates adenylate cyclase activity and that these effects are reversed when G_s is reconstituted into cyc$^-$ cells (Katada *et al.*, 1984b). Similar studies with partially purified catalytic unit from rat brain have shown that both α_i and $\beta\gamma$ subunits possess the ability to inhibit the catalytic unit of adenylate cyclase directly and that their actions are additive (Katada *et al.*, 1986). A direct inhibitory action of these subunits has not been generally accepted because of the high concentrations required (Katada *et al.*, 1984b; 1986) and the suspicion that the action of the subunits may be mediated by deactivation of α_s that may be contaminating the partially purified preparations of catalytic subunit used to test their action (Smigel *et al.*, 1986). However, Katada *et al.* (1987b) have demonstrated that lower and more physiological concentrations of $\beta\gamma$ subunits inhibit the catalytic unit when adenylate cyclase is stimulated by calmodulin in the presence of calcium. The $\beta\gamma$ subunits apparently inhibit adenylate cyclase by binding to calmodulin and thus attenuating its stimulatory action on the enzyme (Katada *et al.*, 1987b). Evidence against hormonal inhibition of adenylate cyclase being mediated by suppression of α_s by free $\beta\gamma$ subunits from G_i comes from the findings of Toro *et al.* (1987). By using cholera toxin treatment to decrease the affinity of

α_s for $\beta\gamma$, and thus its susceptibility to inhibition by $\beta\gamma$, these investigators demonstrated that $\beta\gamma$ subunits are not responsible for the inhibition of adenylate cyclase by somatostatin in GH_3 cells. Although the absolute levels of enzyme activity were elevated in the cholera toxin-treated GH_3 cells, no difference in the dose-response curves for the inhibition of adenylate cyclase by somatostatin was observed between the control and treated cells (Toro *et al.*, 1987). Thus, this study suggests that the catalytic unit of adenylate cyclase is likely to be the target for the inhibitory actions of α_i and/or $\beta\gamma$ subunits.

The $\beta\gamma$ subunits of G proteins have also been reported to have other regulatory roles: They enhance receptor-G protein coupling, GDP/GTP exchange (Correze *et al.*, 1987) and GTPase actvity in reconstituted stimulatory and inhibitory adenylate cyclase systems (reviewed by Hekman *et al.*, 1987). Reconstitution studies show that $\beta\gamma$ dissociated from α_i suppresses basal adenylate cyclase activity thereby enhancing the effects of stimulatory agonists acting through α_s (Cerione *et al.*, 1985). GTPγS binding to turkey erythrocyte α_i (Hekman *et al.*, 1987; Im *et al.*, 1988) and coupling of muscarinic cholinergic receptors to bovine brain α_i and α_o (Florio and Sternweis, 1985) are both enhanced by $\beta\gamma$ subunits. Preliminary studies indicate that interaction of $\beta\gamma$ with the receptor occurs when it is separated from the α subunit (Im,

unpublished, Hekman *et al.*, 1987) and that GDP-bound free α_s subunit is unable to interact with the receptor (Spiegel, 1987).

Both direct inhibitory and stimulatory effects of $\beta\gamma$ subunits have also been reported *in vitro* with other effector systems, such as retinal phospholipase A_2 (Jelsema and Axelrod, 1987) and the cardiac atrial myocyte K^+ channels activated by muscarinic receptors (Logothetis *et al.*, 1987a). However, the physiological significance of these actions of the $\beta\gamma$ subunits remains to be established and in the last instance has been actively disputed (Birnbaumer and Brown, 1987; Logothetis *et al.*, 1987b).

For a signal to be transferred by a G protein from the activated receptor to the catalytic unit of adenylate cyclase, GDP must be replaced by GTP on either G_s or G_i. This could occur by the actual replacement of GDP by GTP or as has been recently suggested, by the conversion of GDP to GTP by a nucleoside diphosphokinase, via the transfer of a high energy phosphate from ATP (Ohtsuki and Yokoyama, 1987; Jakobs *et al.*, 1987; Marquetant *et al.*, 1987). In human platelet and S49 lymphoma membranes, preliminary studies with substrates and inhibitors of nucleoside diphosphokinase indicate that activation of this enzyme by the hormone-receptor complex may be responsible for GTP bound to the G protein (Jakobs *et al.*, 1987; Marquetant *et al.*, 1987).

1.3.3. *Properties of the catalytic unit of adenylate cyclase*

The catalytic unit (C) of adenylate cyclase has proved very difficult to isolate in comparison to the receptor and G protein components of this enzyme system. This has been due to a number of problems including the very low amounts of C present (< 0.01% of the total membrane protein), poor thermal stability upon solubilization in the absence of G_s and the difficulty in reconstituting this subunit in phospholipid vesicles (reviewed in Monneron *et al.*, 1987). Despite these drawbacks, partially purified preparations of C have been isolated by affinity chromatography using a Sepharose-coupled derivative of forskolin (Pfeuffer *et al.*, 1985a,b; Smigel, 1986) or, with brain adenylate cyclase (Coussen *et al.*, 1985; Yeager *et al.*, 1985), calmodulin coupled to Sepharose. A combination of both ligands has also been used with the enzyme from brain (Monneron *et al.*, 1987). The C unit has been purified from rabbit heart (Pfeuffer *et al.*, 1985a), synaptosomes (Coussen *et al.*, 1985) and bovine brain membranes (Yeager *et al.*, 1985; Pfeuffer *et al.*, 1985b). Although use of forskolin or calmodulin yields a 135-155 kDa polypeptide, differences in specific activity (~2 orders of magnitude lower with calmodulin purification) and in responsiveness to calmodulin or preactivated G_s were found. These observations combined with the presence of polypeptides smaller than 135 kDa in fractions with adenylate cyclase activity, have suggested either proteolysis or the presence of more

than one type of C unit in brain adenylate cyclase. The activity of the brain C unit is directly stimulated by forskolin (Seamon *et al.*, 1981), by calmodulin in the presence of physiological Ca^{2+} concentrations and by activated α_s (Smigel, 1986).

1.3.4. *Organization of receptors, G proteins and the catalytic unit of adenylate cyclase in the plasma membrane*

The C unit of adenylate cyclase is probably a transmembrane glycoprotein as it can bind wheat germ agglutinin (Smigel, 1986) and has a catalytic site facing the cytosol that converts ATP into cAMP and pyrophosphate. Although G_s and G_i are considered to be intrinsic membrane proteins because they require solubilization with detergents for their removal from the membrane, they do not appear to be embedded in the plasma membrane. The hydrophilic α subunits appear to be attached to the membrane by the $\beta\gamma$ subunits (reviewed in Neer and Clapham, 1988). In addition, myristoylation of α_i and α_o (but not α_s and α_T) may link them to the hydrophobic region of the $\beta\gamma$ subunits or may attach these proteins directly to the membrane and prevent their release into the cytosol upon dissociation (Buss *et al*, 1987). The importance of the membrane to the functioning of the adenylate cyclase system is demonstrated by studies showing that reconstitution of GTP-dependent hormone-sensitive adenylate cyclase requires phospholipid and that α_{41}, α_{40} and α_{39} subunits are not normally released

from the membrane upon activation, as is α_T (Baehr *et al.*, 1982) and possibly α_s (Lynch *et al.*, 1986). However, upon review of the similarities of the G protein α subunits with α_T and the conditions for protein-protein interactions, Chabre (1987) concludes that G protein interactions with both receptors and effectors occur in the cytosol and that the membrane serves primarily to orientate and concentrate the components involved.

Both the stimulatory and inhibitory receptors that couple to adenylate cyclase are integral membrane proteins that have a high degree of homology to other receptors coupled to G proteins, including rhodopsin in the retinal rod (reviewed by Lefkowitz and Caron, 1988). The best characterized receptor is that of bacteriorhodopsin of *Halobacterium halobium*. High resolution electron diffraction has established the topographical organization of this receptor in the membrane (Henderson and Unwin, 1975). Rhodopsin, adrenergic receptors (β_1, β_2, α_1, α_2) and the four subtypes of muscarinic cholinergic receptors also appear to have a similiar structure based on a number of physical measurements, hydropathicity profiles and biochemical studies (reviewed in Lefkowitz and Caron, 1988). As the abundance of adrenergic receptors in the membrane is quite low, the isolation and sequencing of the genes and/or cDNA clones for the β_1, β_2 and α_2-adrenergic receptors, combined with *in vitro* mutagenesis and proteolysis studies, has allowed testing of the model of topographical organization of receptors based

on bacteriorhodopsin. The evidence suggests that these receptors contain seven transmembrane α helices which create the binding site for the ligand and two less well conserved cytoplasmic loops that may be involved in the coupling of the receptor to the G protein (reviewed by Lefkowitz and Caron, 1988). Through site-directed mutagenesis and amino acid substitution in the cytoplasmic portion of the human β_2-adrenoceptor, O'Dowd *et al.* (1988) have localized the G protein binding site of the receptor to the C terminal end of the third cytoplasmic loop and the N terminal of the cytoplasmic tail.

The activity of the β-adrenergic receptor is also regulated by phosphorylation of serine residues by the cAMP-dependent protein kinase, PKC and the β-adrenergic receptor kinase (reviewed by Sibley *et al.*, 1988). Receptor phosphorylation regulates the stimulation of adenylate cyclase activity by uncoupling the receptor from G_s and, consequently, decreasing enzyme activity. After a prolonged incubation with agonist, hormonal stimulation of adenylate cyclase by a second dose of agonist is much reduced. Homologous densensitization is agonist-specific and is associated with receptor phosphorylation by β-adrenergic receptor kinase followed by receptor sequestration and internalization. Heterologous desensitization results from the phosphorylation of the β-adrenergic receptor by both cAMP-dependent protein kinase and PKC and possibly the phosphorylation of G_s. This type of

desensitization is not specific for the agonists and consequently diminishes the responsiveness of adenylate cyclase to other agonists, guanine nucleotides or NaF.

1.4. *Activation of phospholipase C*

Many cellular processes, including secretion from platelets and neutrophils, are initiated or facilitated by rapid and transient increases in $[Ca^{2+}_i]$ caused by the occupation of specific Ca^{2+}-mobilizing receptors by physiological agonists (reviewed by Abdel-Latif, 1986; Putney, 1987). The resting cell contains an internal Ca^{2+} concentration of about 100 nM, whereas the external Ca^{2+} concentration is in the mM range. Upon activation, the $[Ca^{2+}_i]$ rises very rapidly to 1-10 μM. Since prolonged high concentrations of Ca^{2+} are lethal to the cell, very intricate regulatory mechanisms exist to maintain Ca^{2+} homeostasis. Although by 1975 the extracellular medium, membrane phospholipids and internal Ca^{2+} stores (endoplasmic reticulum or the dense tubular system in the case of the platelet) were known to be reservoirs for Ca^{2+}, very little progress had been made in determining how extracellular signals alter the $[Ca^{2+}_i]$ in cell activation. However, over 30 years ago, Hokin and Hokin (1955) made a discovery that triggered the research that uncovered the signal transduction pathway for Ca^{2+} mobilization. These investigators found that acetylcholine increased the ^{32}P-labelling of membrane phospholipids in the pancreas (Hokin

and Hokin, 1953) and brain slices (Hokin and Hokin, 1955). Although this preliminary finding suggested that the turnover of phosphate in membrane phospholipids was important to hormone action (Hokin and Hokin, 1955), it was some time before the universality of the hormone-stimulated metabolism of phosphoinositides was recognized (Hokin-Neaverson, 1974; Jones and Michell, 1974) and proposed to a be a receptor-mediated event associated with the mobilization of Ca^{2+} (Michell, 1975). PI metabolism does not play a role, however, in the opening of voltage-dependent Ca^{2+} channels, as originally thought (reviewed by Berridge, 1987).

1.4.1. *Substrates and products*

Although the early studies that used neutral chloroform--methanol to extract the phospholipids only detected decreases in PI and increases in PA, extraction with acidified methanol-chloroform later allowed detection of more rapid decreases in PIP_2 upon occupation of hormone receptors (reviewed by Abdel-Latif, 1986). In [^{3}H]inositol-labelled iris smooth muscle, where this reaction was first studied in detail, decreases in [^{3}H]PIP_2 were found to correspond to increases in [^{3}H]IP_3, suggesting that hormone-stimulated PIP_2 breakdown was mediated by activation of phospholipase C (Aktar and Abdel-Latif, 1980). This enzyme breaks the phosphodiester bond in PI and the polyphosphoinositides (PIP and PIP_2) to form DG and the corresponding inositol phosphates (IP,

IP_2 and IP_3) (see Fig. 1.2). These phospholipids, which are thought to reside on the cytoplasmic side of the plasma membrane, constitute less that 8% of total membrane phospholipid in eukaryotic cells and are substrates for specific kinases and phosphomonoesterases that add and subtract phosphate from the inositol group (reviewed by Abdel-Latif, 1986). Newly synthesized PI is transported from the endoplasmic reticulum to the plasma membrane. There it is converted to PIP by PI kinase, which transfers phosphate from ATP to the fourth hydroxyl residue on the inositol ring (Berridge, 1987). In turn, PIP is converted to PIP_2 by a distinct PIP kinase, which transfers a phosphate to the fifth hydroxyl residue on the inositol ring. These polyphosphoinositides are found in much smaller amounts in the membrane than PI and are metabolized more rapidly (Berridge, 1987). Accumulating evidence from a variety of cells suggests that receptor-mediated activation of phospholipase C initially produces DG and IP_3 from PIP_2 (Berridge and Irvine, 1984) though, in the platelet, PIP may also be a major substrate of the enzyme (Culty *et al.*, 1988). Both DG and IP_3 have now been shown to be the second messengers responsible for mediating a wide range of physiological events (Nishizuka, 1984; Berridge and Irvine, 1984).

In platelets labelled with ^{32}P and/or [^{3}H]AA, thrombin (Siess *et al.*, 1984; Rittenhouse, 1984), TxA_2 mimetics (Rittenhouse, 1984; Siess *et al.*, 1983), PAF (Siess *et al.*,

1984), AVP (Siess *et al.*,1986) and collagen (Pollock *et al.*, 1986), directly stimulate the formation of [^{32}P]PA and [^{3}H]DG. In the thrombin-stimulated platelet, as in other cells, decreases in PI (Rittenhouse-Simmons, 1979), PIP and PIP_2 (Billah and Lapetina, 1982) and corresponding increases in IP, IP_2 and IP_3 (Agranoff *et al.*, 1983) have been observed (reviewed by Haslam, 1987). Since PIP_2 degradation occurs within seconds and is neither dependent on increases in $[Ca^{2+}_i]$ nor induced by the Ca^{2+} ionophore A23187 (Rittenhouse, 1984), as is the slower degradation of PI (Wilson *et al.*, 1985), PIP_2 has been thought to be the preferred substrate of hormone-stimulated phospholipase C. This hypothesis is supported by the rapid formation of IP_2 and IP_3 with initially a much smaller and slower accumulation of IP (Siess, 1985; Siess *et al.*, 1986; Shukla, 1985). The high levels of IP_2 formed in many cells were thought to reflect the rapid degradation of IP_3 to IP_2 by a IP_3 5'-phosphatase. However, in rabbit platelet (Hrbolich *et al.*, 1987) and GH_3 cell membranes (Straub and Gershengorn, 1986; Martin *et al.*, 1986), PIP rather than PIP_2 appears to be the predominant substrate of phospholipase C. The same was also found in permeabilized human platelets at a pCa of 6 (Culty *et al.*, 1988). The large decreases in PI in intact thrombin-stimulated platelets have not been satisfactorily explained by the following pathways; (1) conversion of PI into PIP and PIP_2 by their specific kinases or (2) the action of PLA_2 to form lyso-PI (reviewed in Haslam, 1987). It is possible that strong

stimuli, such as thrombin, also initiate a Ca^{2+}-stimulated hydrolysis of PI in platelets (Siess, 1985; Culty *et al.*, 1988). As a result of the compartmentation, rapid hydrolysis and interconversion of phosphoinositides, definitive identification of the major substrate of receptor-mediated activation of phospholipase C in intact platelets has yet to be obtained.

With the more recent use of HPLC instead of Dowex 1 resin to separate [^{3}H]inositol phosphates from cells prelabelled with [^{3}H]inositol, various positional isomers of [^{3}H]IP, [^{3}H]IP_2, [^{3}H]IP_3 and [^{3}H]inositol tetrakis- and pentakisphosphates have been discovered (reviewed in Berridge, 1987; Majerus *et al.*, 1988). Although more than one phospholipase C exists in most cells, each enzyme appears to be able to convert PI, PIP and PIP_2 to their respective inositol 1-phosphate and cyclic (1:2)-phosphate counterparts (see Fig. 1.2). These products are then phosphorylated or dephosphorylated by kinases and phosphomonoesterases. Of all the compounds identified, inositol 1,4,5-trisphosphate is the only inositol phosphate that has the characteristics of a second messenger molecule and mediates Ca^{2+} mobilization in a wide variety of cells (reviewed in Berridge, 1987). Inositol 1,4,5-trisphosphate rapidly increases $[Ca^{2+}_i]$ by stimulating the efflux of Ca^{2+} from components of the endoplasmic reticulum in most cells (Berridge, 1987) and probably from the dense tubular system in platelets (O'Rourke *et al.*, 1985, 1987; Brass and Joseph, 1985).

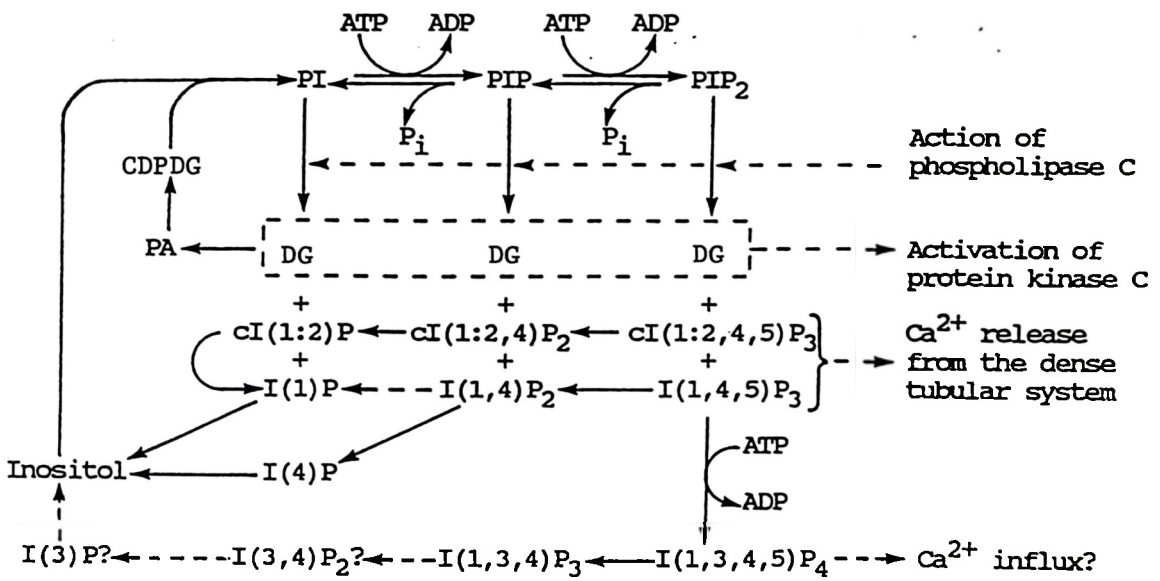
Preliminary investigations suggest that inositol 1,3,4,5-tetrakis-phosphate formed by the phosphorylation of inositol 1,4,5-trisphosphate may facilitate agonist-induced Ca^{2+} influx across the plasma membrane (reviewed by Berridge, 1987). Inositol 1,3,4-trisphosphate formed from the dephosphorylation of inositol 1,3,4,5-tetrakisphosphate has been postulated to release Ca^{2+} from internal stores during the later and more prolonged phase of the Ca^{2+} response (reviewed by Berridge, 1987).

1.4.2. *The PI cycle*

The breakdown and resynthesis of the membrane PI and polyphosphoinositides has been referred to as the PI cycle (reviewed in Berridge and Irvine, 1984; see Fig.1.2). Upon occupation of Ca^{2+} mobilizing receptors by strong agonists, PIP_2, PIP and possibly PI are diverted from futile cycles of phosphorylation/dephosphorylation and hydrolysed by phospholipase C to produce DG and the various inositol phosphates. PI is then resynthesized by the following reactions; DG is converted to PA by DG kinase and thence to cytidine diphosphate-DG by cytidine triphosphate phosphatidate cytidyl transferase. Cytidine diphosphate-DG and free inositol, the latter produced by the dephosphorylation of inositol phosphates by specific phosphatases, are finally converted to PI by cytidine diphosphate-DG inositol phosphatidate transferase.

Fig. 1.2. Metabolism of phosphoinositides and inositol phosphates in platelets

Abbreviations; CDPDG, CDP-diacylglycerol; cI(1:2)P, inositol 1:2-cyclic monophosphate; cI(1:2:4)P_3, inositol 1:2-cyclic, 4-bis-phosphate; cI(1:2,4,5)P_3, inositol 1:2 cyclic, 4,5-trisphosphate; I(1)P, inositol 1-phosphate; I(1,4)P_2, inositol 1,4-bisphosphate; I(1,4,5)P_3, inositol 1,4,5-trisphosphate; I(4)P, inositol 4-phosphate; I(3)P, inositol 3-phosphate; I(3,4)P_2, inositol 3,4-bisphosphate; I(1,3,4)P_3, inositol 1,3,4-trisphosphate; I(1,3,4,5)P_4, inositol 1,3,4,5-tetrakisphosphate. All phosphoinositides and inositol phosphates have the D-myo-inositol configuration. This figure and abbreviations in the legend are taken from Haslam (1987).



1.4.3. *Role of guanine nucleotides*

In addition to the role of guanine nucleotides in the hormonal stimulation and inhibition of adenylate cyclase (Spiegel, 1987) and in the stimulation of cGMP phosphodiesterase in the retinal rod (Bitensky *et al.*, 1984), several lines of evidence support the involvement of G-proteins in agonist-stimulated phosphoinositide metabolism (Litosch and Fain, 1986). Early studies with a number of different cell types demonstrated that the affinities for agonists of receptors coupled to Ca^{2+} mobilization were decreased in the presence of guanine nucleotides (Goodhardt *et al.*, 1982; Snyderman *et al*, 1984). Experiments with permeabilized mast cells (Gomperts, 1983) demonstrated that the intracellular incorporation of GTP analogues increased the secretion of histamine from these cells. Since secretion is dependent on extracellular Ca^{2+}, Gomperts (1983) postulated that a distinct G protein may regulate Ca^{2+} channels in the plasma membrane in mast cells. However, studies with permeabilized human platelets (Haslam and Davidson, 1984a,b,c), which demonstrated guanine nucleotide-dependent secretion of [^{14}C]5-HT, suggested that Ca^{2+} mobilizing receptors were coupled to phosphoinositide breakdown by a G protein. In permeabilized platelets, GTP analogues increased secretion of [^{14}C]5-HT and reduced the requirement of this process for [Ca^{2+}_{free}] to physiological levels (pCa 7). Furthermore, secretion caused by stable analogues of GTP was associated with

increased formation of [^{3}H]DG (Haslam and Davidson, 1984a,c) and [^{3}H]inositol phosphates (Culty *et al.*, 1988) in permeabilized platelets labelled with [^{3}H]AA or [^{3}H]inositol, respectively. These guanine nucleotides acted synergistically with thrombin to stimulate the secretion of [^{14}C]5-HT and formation of [^{3}H]DG and [^{3}H]inositol phosphates (Haslam and Davidson, 1984,b,c; Haslam *et al.*, 1985; Culty *et al.*, 1988), suggesting that a G protein is involved in the activation of phospholipase C by thrombin. Further early support for a role for a G protein in the coupling of Ca^{2+} mobilizing receptors to phosphoinositide breakdown came from the studies of permeabilized rat pancreatic acinar cells (Merritt *et al.*, 1986) and permeabilized GH_3 cells (Martin *et al.*, 1986).

Studies using cell-free systems have permitted a more detailed characterization of the guanine nucleotide-dependent hormonal activation of phospholipase C. Studies with plasma membrane preparations from neutrophils (Cockcroft and Gomperts, 1985), rat cerebral cortex (Gonzales and Crews, 1985) and rat hepatocytes (Wallace and Fain, 1985) demonstrated that stable analogues of GTP caused decreases in polyphosphoinositides or increases in inositol phosphates. Direct evidence for the involvement of a G protein in the agonist-stimulated activation of phospholipase C was first obtained with the demonstration of a GTP-dependent stimulation of inositol phosphate formation by 5-HT in blowfly salivary gland membranes (Litosch *et al.*, 1985). Since then, a GTP requirement

for activation of phospholipase C by agonists has been demonstrated in a number of other cell and tissue membrane preparations (Litosch and Fain, 1986), including platelets (Baldassare and Fisher, 1986; Hrbolich *et al.*, 1987). In studies that measured both the breakdown of polyphosphoinositides and formation of inositol phosphates caused by agonists in the presence of guanine nucleotides, decreases in PIP_2 (Uhing *et al.*, 1986, Lucas *et al.*, 1985, Taylor and Exton, 1987) and/or PIP (Lucas *et al.*, 1985; Uhing *et al.*, 1986; Hrbolich *et al.*, 1987) were accounted for by increases in IP_3 and IP_2.

1.4.4. *Identification of the G protein mediating activation of phospholipase C by agonists*

To determine whether the G protein(s) thought to be involved in hormonal inhibition of adenylate cyclase (G_i) also couple Ca^{2+}-mobilizing receptors to phospholipase C, many cell types and membranes prepared from them have been treated with pertussis toxin. Treatment with this toxin suppressed activation of phospholipase C by agonists in some cells but not others. Pertussis toxin-sensitive systems included phospholipase C coupled to chemoattractant receptors in HL-60 cells (Brandt *et al.*, 1985), human (Smith *et al.*, 1985; Krause *et al.*, 1985), guinea pig (Okajima *et al.*, 1985) and rabbit neutrophils (Volpi *et al*, 1985) and human monocytes (Verghese *et al.*, 1986). Phospholipase C coupled to TxA_2

receptors in rat mast cells (Nakamura and Ui, 1985), α_1-adrenergic receptors in rat fat cell membranes (Rapiejko *et al.*, 1986) and cultured rat myocytes (Steinberg *et al.*, 1987), thrombin receptors in hamster fibroblastic CCL39 cells (Paris and Pouyssegur, 1986) and bradykinin receptors in clonal F-11 dorsal root ganglion hybrid cells (Francel *et al.*, 1987) were also affected. However, pertussis toxin treatment had no effect on phosphoinositide hydrolysis caused by carbachol in cultured chicken heart (Masters *et al.*, 1985) and 1321N1 human astrocytoma cells (Masters *et al.*, 1985; Hepler and Harden, 1986), by thyrotropin-releasing hormone in permeabilized GH_3 cells (Martin *et al.*, 1986) and GH_3 pituitary cell membranes (Straub and Gershengorn, 1986), by caerulein in permeabilized rat pancreatic acinar cells (Merrit *et al.*, 1986), by angiotensin II, AVP and epinephrine in rat liver plasma membranes (Uhing *et al.*, 1986), and by thrombin, bradykinin and PAF in mouse 3T3 fibroblasts (Murayama and Ui, 1985). In the platelet, activation of phospholipase C by thrombin was attenuated by pertussis toxin in some studies (Brass *et al.*, 1986; O'Rourke *et al.*, 1987) and increased in others (Lapetina, 1986a). In some cell systems, the pertussis toxin sensitivity of phospholipase C activation depended on the type of receptor coupled to this effector. For example, in hepatocytes, the activation of phospholipase C by EGF was blocked by pertussis toxin treatment, whereas stimulation of this enzyme by angiotensin II was pertussis toxin-insensitive (Johnson and Garrison,

1987). Differences in the pertussis toxin sensitivity of Ca^{2+}-mobilizing receptors have also been observed in human platelets (Brass *et al.*, 1986). The pertussis toxin sensitivity of agonist-induced activation of phospholipase C also appears to be dependent on the type of cell. For example, activation of phospholipase C by angiotensin II was pertussis toxin-sensitive in renal mesangial cells, but was pertussis toxin-insensitive in hepatocytes (Becker *et al.*, 1987).

1.4.5. *Characterization of phosphoinositide-specific phospholipase C*

Multiple isoforms of Ca^{2+}-dependent phosphoinositide-specific phospholipase C have been isolated from a number of cells and tissues including bovine (Lee *et al.*, 1987; Ryu *et al.*, 1987; Katan and Parker, 1987) and rat (Irvine *et al.*, 1984; Kosawa *et al.*, 1987) brain, human platelets (Hakata *et al.*, 1982; Banno *et al.*, 1986; Low *et al.*, 1986), sheep seminal vesicles (Hofmann and Majerus, 1982), porcine lymphocytes (Carter and Smith, 1987) and murine thymocytes (Wang *et al.*, 1986). Several phosphoinositide-specific phospholipase C isoforms appear to be cytosolic and to date, five immunologically distinct species have been identified (reviewed by Ryu *et al.*, 1987). In addition, phosphoinositide-specific phospholipase C isoforms have been isolated from particulate fractions of rat brain (Kozawa *et al.*, 1987), bovine brain (Lee *et al.*, 1987; Katan and Parker, 1987), human

platelets (Banno and Nozawa, 1987; Banno *et al.*, 1988), porcine lymphocytes (Carter and Smith, 1987) and murine thymocytes (Wang *et al.*, 1986). In most studies, PIP_2 was the prefered substrate of the membrane-bound enzyme at low Ca^{2+} concentrations, suggesting that this enzyme may be involved in receptor-mediated phosphoinositide hydrolysis. It is likely that these isoenzymes of phospholipase C have different functions *in vivo* because of their differential distribution in mammalian cells (Low *et al.*, 1984) and their unique biochemical and kinetic properties (Ryu *et al.*, 1987). The recent cloning and sequencing of four of the phosphoinositide-specific phospholipase C isoenzymes has shown that they possess little homology with each other and that their activities are likely to be regulated by different mechanisms (Stahl *et al.*, 1988; Bennett *et al.*, 1988; Suh *et al.*, 1988a,b; Katan *et al.*, 1988).

In the platelet and in other cells, the enzyme involved in GTP-dependent activation of phospholipase C by agonists remains to be defined. Since most of the platelet phospholipase C activity is cytosolic (Rittenhouse, 1983) and addition of platelet cytosol appeared to increase the activation of phospholipase C by $GTP\gamma S$ added either alone or with thrombin (Baldassare and Fisher, 1986), it is possible that phosphoinositide hydrolysis is mediated by the interaction of activated G proteins with the soluble enzyme. In support of this hypothesis, enhanced degradation of $[^3H]PIP_2$ in

phospholipid vesicles was observed on addition of platelet supernatant and guanine nucleotides, including GTPγS (Deckmyn *et al.*, 1986; Banno *et al.*, 1986). Moreover, G proteins have been detected in the cytosol of platelets (Deckmyn *et al.*, 1986; Wang *et al.*, 1987) and mast cells (Katada and Ui, 1982). To date, three phosphoinositide-specific phospholipase C enzymes of M_r 67,000 (Banno *et al.*, 1986), 95,000 and 140,000 (Hakata *et al.*, 1982; Low *et al.*, 1986) have been isolated from the platelet cytosol and a GTPγS-binding protein of 27 kDa has been stated to co-purify with soluble phospholipase C from human platelets and to increase [^{3}H]PIP_2 hydrolysis by the purified enzyme (Wang *et al.*, 1987; 1988). However, it is possible that platelet lysis and fractionation disrupts the normal distribution of the G proteins and phospholipase C that are involved in receptor-mediated phosphoinositide breakdown and releases membrane-bound proteins into the cytosol. Attempts to address this question in rabbit platelet membranes, demonstrated that activation of membrane-bound phospholipase C by thrombin and GTP was only decreased slightly after being washed with hypotonic buffer containing 1 M KCl (Haslam *et al.*, 1988a). Furthermore, addition of dialysed platelet cytosol to washed platelet membrane preparations increased both the GTPγS and thrombin and GTP-stimulated [^{3}H]inositol phosphate formation by less than 50% (Haslam *et al.*, 1988a). A phospholipase C with molecular mass of 110 kDa, that catalyses the hydrolysis of PIP_2 more efficiently than PI at physiological concentrations of

Ca^{2+}_{free} was solubilized and partially purified from human platelet membranes (Banno and Nozawa, 1987). This enzyme was stimulated by purified rat brain G_i or G_o (Banno *et al.*, 1987). Recently, two PLC isoenzymes that prefer PIP_2 as their substrate have been isolated from human platelet membranes (Banno *et al.*, 1988). These latter studies suggest that soluble phospholipase C does not play a major role in signal transduction in platelets and that stimulation of phosphoinositide hydrolysis by agonists and guanine nucleotides is mediated by proteins firmly bound to the plasma membrane.

1.5. *PKC*

Since its identification in 1977 (Takai *et al.*, 1977; Inoue *et al.*, 1977), PKC has been shown to play very important roles in signal transduction. It not only mediates a number of cellular processes but also regulates these events through both positive and negative feedback mechanisms (Nishizuka, 1984; Kikkawa and Nishizuka, 1986). This widely distributed enzyme phosphorylates serine and threonine but not tyrosine residues on a large number of proteins from a variety of tissues (Nishizuka, 1984). Although some of these phosphoproteins have been identified, the functions of others in the biological actions of PKC remain to be determined.

1.5.1. *Regulation of PKC*

This enzyme can be activated by at least three different mechanisms *in vitro*. In the presence of Ca^{2+} and phospholipid, DG produced from the breakdown of the phosphoinositides stimulates PKC. This neutral lipid acts to increase the affinity of PKC for Ca^{2+} and phospholipid and, consequently, induces maximal enzyme activity at physiological Ca^{2+} concentrations (0.1-1 μM) (Kishimoto *et al.*. 1980). Together, Ca^{2+} and DG augment PKC activity synergistically (Nishizuka, 1984). Thus, Nishizuka and coworkers (Kishimoto *et al.*, 1980; 1984) proposed that DG, as well as Ca^{2+}, acts as a second messenger to mediate the effects of receptor-activated phosphoinositide breakdown. DG is not detectable in plasma membranes of resting cells and, once formed, is rapidly phosphorylated to form PA or hydrolysed by DG and monoglyceride lipases to release AA (Prescott and Majerus, 1983). Although the second messenger role of DG is transient in nature, the effect of activation of PKC may be prolonged, as the incorporation of phosphate into PKC substrates is often quite resistent to phosphoprotein phosphatases (Kikkawa and Nishizuka, 1986). The requirements for phospholipid and DG are specific, with phosphatidylserine and *sn*-1,2 DGs being the most effective activators. *sn*-1-Oleoyl-2-acetyl-glycerol (Kaibuchi *et al.*, 1983; Watson and Lapetina, 1985; Lapetina *et al.*, 1985) and DGs containing saturated acyl chains with six, eight or ten carbon atoms (Lapetina

et al., 1985) have been used to study the actions of PKC since they can penetrate intact cells.

Several lines of evidence strongly suggest that PKC is the intracellular receptor for phorbol esters and that the cellular actions of these tumor promoters are mediated by the activation of this enzyme (Kikkawa and Nishizuka, 1986). These compounds have been valuable in delineating the importance of the DG/PKC pathway in a number of cellular processes because of their ability to mimic the action of endogenous DG. In addition, these compounds are very potent (active at submicromolar concentration) because of their resistance to degradation and are able to bypass receptor-mediated increases in DG (Kikkawa and Nishizuka, 1986). Activation of PKC by incubating intact cells with phorbol ester, synthetic DG or Ca^{2+}-mobilizing agonists is associated with decreases in cytosolic PKC and increases in membrane-bound PKC. Binding of PKC to the plasma membrane is a Ca^{2+}-dependent process that requires increases in Ca^{2+}_i above 100-200 nM (Wolf *et al.*, 1985). Removal of Ca^{2+} releases PKC into the cytosol (Wolf *et al.*, 1985). PMA decreases the Ca^{2+} requirement for PKC binding to the plasma membrane and blocks the release of PKC upon removal of Ca^{2+} (Wolf *et al.*, 1985). Translocation of the enzyme serves to bring PKC in contact with membrane substrates.

PKC can also be activated by soluble Ca^{2+}-dependent neutral

thiol proteases called calpains in a reaction that is enhanced by phosphatidylserine and DG (Kishimoto *et al.*, 1983). These Ca^{2+}-dependent proteases stimulate the active membrane-bound form of PKC. The Ca^{2+}, phosphatidylserine and DG-dependent PKC of 77 kDa is converted by limited proteolysis to a Ca^{2+} and phosphatidylserine-independent, irreversibly activated 50 kDa enzyme (Inoue *et al.*, 1977; Tapley and Murray, 1985). Although the physiological significance of this reaction remains uncertain, as it is dependent on a fairly high $[Ca^{2+}_i]$ and has not been observed with physiological agonists, it has been proposed to play a role in the activation of PKC in human platelets (Tapley and Murray, 1984a,b; 1985) and neutrophils (Melloni *et al.*, 1985). Calpain I is abundant in human platelets (Tsujinaka *et al.*, 1982) and manifests its proteolytic activity at a $[Ca^{2+}_{free}]$ in the μM range (Kishimoto *et al.*, 1983). As with PKC, the Ca^{2+}-dependent translocation of calpain from the cytosol to the plasma membrane of the neutrophil decreases the requirement of Ca^{2+}_i for enzyme activation (Melloni *et al.*, 1985). Thus, the proteolytic activity of calpain is seen at a physiological $[Ca^{2+}_i]$ when the enzyme is in the membrane-bound form. As the PMA-induced activation of PKC in human platelets was blocked by leupeptin, Tapley and Murray (1985) have proposed that the activation of PKC proceeds via the Ca^{2+}-dependent transfer of cytosolic PKC and calpain to the membrane. In support of this hypothesis, is the detection of the Ca^{2+} and phosphatidylserine-independent fragment

of PKC in the cytosol in PMA-treated human platelets (Tapley and Murray, 1984b). A specific role for calpain in mediating the action of thrombin has been suggested (Ruggerio and Lapetina, 1986), but this was based on effects of inhibitors that directly block the proteolytic activity of thrombin itself (Brass and Shattil, 1988). Samis *et al.* (1987) have shown with a monoclonal antibody to calpain I that this protease is not cleaved to its active form when platelets are stimulated by thrombin without stirring. However, when thrombin-activated platelets were stirred to induce aggregation, the active form of calpain could be detected. Thus, a physiological role for Ca^{2+}-dependent proteases in the initial activation of platelets has not been generally accepted.

The third mechanism proposed for activation of PKC that is gaining increasing recognition, especially in specialized cells, is the direct stimulation of the enzyme by unsaturated free fatty acids (reviewed in Dreher and Hanley, 1988). AA which can be released from DG by diacyl and monoacylglycerol lipases (Prescott and Majerus, 1987) or from membrane phospholipids upon activation of PLA_2 (Lapetina, 1981), was initially proposed as the second messenger for a novel pathway of Ca^{2+} and phospholipid-independent activation of PKC in brain (Murakami and Routtenberg, 1985). Related compounds could also be involved, since oleic acid (Murakami *et al.*, 1986) and an AA metabolite, lipoxin A (Hansson *et al.*, 1986), can also activate PKC *in vitro*. However, direct evidence of a

physiological role for this pathway in receptor-mediated activation of PKC is still lacking (reviewed by Dreher and Hanley, 1988). Discrepancies in the action of AA in different tissues are due to the presence of different isoforms of PKC with distinct functions and AA sensitivities (Nishizuka, 1988). At least seven isoforms of PKC (Ono *et al.*, 1987) have been identified, two of which (α and γ) are known to respond to AA (Nishizuka, 1988).

In addition to the above mechanisms for activation of PKC, the activity of this enzyme may also be subject to negative regulation. For example, autophosphorylation of PKC may inhibit its activity, as has been demonstrated with Ca^{2+}-calmodulin-dependent protein kinase type II (LeVine *et al.*, 1985). Autophosphorylation has also been implicated in the release of activated PKC from the membrane, since the release of purified PKC from erythrocyte vesicles in the presence of PMA did not occur on the removal of Ca^{2+} alone but also required ATP and $MgCl_2$ (Wolf *et al.*, 1985). Dreher and Hanley (1988) have also speculated that the PKC isoforms may control the activity of each other by phosphorylation. The activity of PKC may also be regulated by sphingosine (Hannun *et al.*, 1986), gangliosides (Kreutter *et al.*, 1987) and phosphatidylcholine (Kaibuchi *et al.*, 1981), as these compounds attenuate enzyme activity *in vitro*.

1.5.2. *Characterization of PKC*

PKC is a single polypeptide of 77 kDa that is composed of a hydrophobic regulatory domain (~27 kDa) and a hydrophilic catalytic domain (~50 kDa). PKC as first purified from brain by Nishizuka and coworkers (Takai *et al.*, 1977) was found to be a doublet on SDS-polyacrylamide gels and was largely associated with synaptosomal membranes (reviewed by Kikkawa and Nishizuka, 1986). However, in most other cells and tissues, PKC is located in the cytosol in its inactive form (reviewed by Kikkawa and Nishizuka, 1986). To date, three types of PKC separable on hydroxyapatite columns have been purified from brain (Sekiguchi *et al.*, 1987; Kosaka *et al.*, 1988) and two types from human platelets, which correspond biochemically and immunologically to types II (β_I and β_{II}) and III (α) from rabbit brain (Watanabe *et al.*, 1988). In addition, platelets contain a Ca^{2+}-insensitive form of the enzyme (Nishizuka, 1988). Recently, sequencing of PKC cDNA clones from a rat brain library (Ono *et al.*, 1987) has revealed three additional isoforms of PKC that are distinct from types I (γ), II (β_I and β_{II}) and III (α). All isoforms of PKC have the similar structural characteristics, as predicted by sequencing of cDNA clones (Ono *et al.*, 1987; Nishizuka, 1988). The single PKC polypeptide contains a number of conserved (C1-C4) and variable (V1-V5) domains. The regulatory part of PKC, at the NH_2 terminal, contains C1, C2, V1 and V2 domains; these conserved domains

are thought to contain the binding sites for Ca^{2+}, phosphatidylserine and DG (See Nishizuka, 1988). The catalytic carboxy terminal part of the molecule comprises domains C3, C4, V3, V4 and V5 (see Nishizuka, 1988). C3 contains the ATP-binding site and C3 and C4 are critical to the catalytic activity of PKC.

1.5.3. *Role of PKC in platelet activation*

Studies with phorbol esters and synthetic DG, have shown that PKC plays an important role in stimulus-reponse coupling in a number of cells including platelets. Treatment of human platelets with PMA (Yamanishi *et al.*, 1983; Kaibuchi *et al.*, 1983) or a permeable DG such as diC_8 (Lapetina *et al.*, 1985) causes platelet aggregation and secretion but not shape change. Incubation of platelets with concentrations of both the Ca^{2+} ionophore, A23187, and PMA that alone induce little platelet aggregation and secretion causes maximal aggregation and secretion similar to that observed with thrombin (Yamanishi *et al.*, 1983; Kaibuchi *et al.*, 1983). Platelet activation by thrombin and collagen is accompanied by major increases in the phosphorylation of a 40-47 kDa protein (P47) and of the 20 kDa myosin light chain (Lyons *et al.*, 1975; Haslam and Lynham, 1977; Haslam *et al.*, 1979). Although the identity of P47 remains uncertain, it has been variously proposed to be lipocortin, an inhibitor of PLA_2 (Touqui *et al.*, 1986), the α-subunit of pyruvate dehydrogenase (Chiang *et al.*,

1987), the IP_3 5-phosphatase (Connolly *et al.*, 1986) and a regulator of actin polymerization (Hashimoto *et al.*, 1987). The first two of these proposals have been disproved by the recent cloning and sequencing of P47 cDNA (Tyers *et al.*, 1988) and the third has been questioned (Haslam, 1987). This protein has now been renamed pleckstrin (Tyers *et al.*, 1988). Studies with PMA and with purified P47 and PKC have demonstrated that P47 is the major platelet substrate of PKC (Nishizuka, 1984). Myosin light chain is phosphorylated by both the Ca^{2+}/calmodulin-dependent myosin light chain kinase (Adelstein and Conti, 1975; Hathaway and Adelstein, 1979) and PKC, but at different sites (Naka *et al.*, 1983). Nishizuka and colleagues (reviewed in Nishizuka, 1984) have proposed that Ca^{2+} mobilization and activation of PKC act synergistically to cause an optimal platelet response. This model appears to account fully for dense granule secretion (Haslam *et al.*, 1979; Yamanishi *et al.*, 1983) but only partially for lysosomal enzyme secretion (Kajikawa *et al.*, 1983; Knight *et al.*, 1984). Activation of PKC also induces the expression of the platelet fibrinogen receptor, glycoprotein IIb-IIIa (Shattil and Brass, 1987), enhances polyphosphoinositide formation from PI (Halenda and Feinstein, 1984) and stimulates PLA_2 in human platelets (Halenda *et al.*, 1985).

1.5.4. *Regulation of signal transduction by PKC*

Besides promoting stimulus-response coupling, PKC has a major

role in regulating cellular events by feedback mechanisms that mainly curtail production of the second messengers involved in signal transduction. Phorbol esters can inhibit the actions of physiological agonists on both the phospholipase C and adenylate cyclase pathways. Thus, preincubation with PMA has been found to block agonist-induced PI metabolism and Ca^{2+}-mobilization in a wide variety of cells (Abdel-Latif, 1986). In human platelets, PMA decreases thrombin-induced PIP_2 breakdown and inositol phosphate and PA formation (MacIntyre *et al.*, 1985a; Zavoico *et al.*, 1985; Crouch and Lapetina, 1988). Activation of PKC with PMA blocked both the influx of $^{45}Ca^{2+}$ and the increases in $[Ca^{2+}_i]$ caused by PAF, thrombin and U46619 (Avdonin *et al.*, 1987). The effects of PMA on thrombin-stimulated PI metabolism and Ca^{2+} mobilization in human platelets were opposed by the PKC inhibitors, H7 (Tohmatsu *et al.*, 1986) and staurosporine (Crouch and Lapetina, 1988). In contrast, Watson *et al.* (1988) reported that staurosporine had no effect on the activation of PI breakdown by thrombin (Watson *et al.*, 1988). The actions of PMA on agonist-stimulated PI metabolism are selective. In liver (Corvera and Garcia-Sainz, 1984) and Swiss 3T3 cells (Sturani *et al.*, 1986), PMA blocked the the coupling of some Ca^{2+}-mobilizing receptors to phospholipase C but not that of others. The specificity of the inhibitory action of PMA for particular agonists, coupled with a similar time course for this effect and for the phosphorylation of the α_1-receptor in smooth muscle cells (Leeb-Lundberg *et al.*, 1985), suggests that receptor

phosphorylation may mediate these effects of PKC. It is also possible that phosphorylation of IP_3 5-phosphatase by PKC, which has been reported to enhance its activity (Connolly *et al.*, 1986), is responsible for decreased levels of IP_3 and $[Ca_i]$ in PMA-treated cells, including platelets (Zavoico *et al.*, 1985; Rittenhouse and Sasson, 1985). PMA also accelerates Ca^{2+} removal from the cytosol of platelets (Pollock *et al.*, 1987). Bennett and Crooke (1987) have shown that PI-specific phospholipase C is phosphorylated in PMA-treated rat basophilic leukemic cells and that purified PKC phosphorylates partially purified PI-specific phospholipase C from guinea pig uterus. Since the majority of the non-phosphorylated and phosphorylated forms of PI-specific phospholipase C were found in the cytosol and membrane fractions, respectively, these workers have suggested that phosphorylation of the enzyme may regulate its activation and deactivation by controlling its movement within the cell and thus, its interaction with G proteins and receptors that are a part of the phosphoinositide pathway. The G proteins coupling Ca^{2+}-mobilizing receptors to phospholipase C could also be PKC substrates. This hypothesis is supported by the inability of GTPγS to cause PIP_2 breakdown in membranes prepared from PMA-treated leukocytes (Smith *et al.*, 1987). This action of PMA was specific to the G protein because the fMLP-stimulated binding of GTPγS was not blocked and the phospholipase C remained responsive to mM concentrations of Ca^{2+} after PMA treatment (Smith *et al.*, 1987).

In many studies, PMA has also been found to attenuate hormonal inhibition (Jakobs *et al.*, 1985) and stimulation (Garte and Belman, 1980; Heyworth *et al.*, 1984; Kelleher *et al.*, 1984; Kassis *et al.*, 1985; Quilliam *et al.*, 1985) of adenylate cyclase in membrane preparations. Some of these effects may reflect phosphorylation at the receptor level (Nishizuka *et al.*, 1988). However, several groups have also reported increased cAMP formation in response to hormonal stimulation of adenylate cyclase in membranes prepared from PMA-treated cells including platelets (Jakobs *et al.*, 1985; Quilliam *et al.*, 1985; Sugden *et al.*, 1985; Bell *et al.*, 1985; Yoshimasa *et al.*, 1987; Rozengurt *et al.*, 1987; Langlois *et al.*, 1987). Many of these effects have been attributed to phosphorylation of the α subunit of G_i by PKC (Katada *et al.*, 1985), which can both block inhibition of adenylate cyclase and enhance stimulation of the enzyme. The precise effect of PMA appears to depend on the cell, the agonist studied and the experimental conditions (Johnson *et al.*, 1986).

1.6. *Signal transduction pathways mediating platelet activation by different agonists*

The platelet is activated to various degrees by different agonists as a result of the stimulation of a number of distinct effector systems that generate second messengers in different

proportions (Rink and Hallam, 1984; Haslam *et al.*, 1985). All platelet agonists studied interact with specific binding sites on the platelet membrane and cause platelet activation through receptor-mediated events (reviewed by MacIntyre *et al.*, 1986). The actions of the following agonists will be considered to clarify the signal transduction pathways mediating platelet activation; thrombin, AVP, PAF, prostaglandin endoperoxides and TxA_2, ADP and epinephrine. The effects of each of these agonists is enhanced substantially by the concurrent agonist-induced formation of prostaglandin endoperoxides and TxA_2 from AA and by ADP released from the dense granules (Rink and Hallam, 1984; Haslam, 1987). Thus, in order to examine the primary actions of the different platelet agonists, it is necessary to preincubate platelet suspensions with a cyclooxygenase inhibitor and an ADP-scavenging enzyme system, so that the observed platelet responses cannot be accounted for by the production or release of these secondary mediators.

1.6.1. *Thrombin*

Thrombin is generated by the factor Xa-induced cleavage of circulating prothrombin at sites of vascular damage and is responsible for both platelet aggregation and the formation of fibrin from fibrinogen. Of all the known platelet agonists, thrombin causes the widest range of platelet responses, including platelet shape change, aggregation and secretion of the contents of dense, α-

and acid hydrolase-containing granules (Rink and Hallam, 1984; Steen and Holmsen, 1987; Haslam, 1987). Although thrombin stimulates the production of TxA_2, it is not dependent on released TxA_2 for its primary effects (Rink and Hallam, 1984). Platelet activation by thrombin usually results from the combined actions of increased $[Ca^{2+}_i]$ and of DG in the platelet membrane. However, thrombin can induce platelet activation independently of a rise in $[Ca^{2+}_i]$ (Rink *et al.*, 1983). The rise in $[Ca^{2+}_i]$ is a consequence of the ability of thrombin to stimulate both an influx of extracellular Ca^{2+} and the formation of IP_3, which acts to release Ca^{2+} from internal stores (reviewed in Rink and Hallam, 1984; Haslam, 1987). Zschauer *et al.* (1988) have recently shown that thrombin-stimulated human platelets contain voltage-independent Ca^{2+} channels. Activation of phospholipase C by thrombin has been demonstrated in intact platelets by the short-lived decreases in PI (Rittenhouse-Simmons, 1979; Wilson *et al.*, 1985) and polyphosphoinositides (Billah and Lapetina, 1982; Agranoff *et al.*, 1983) which are associated with transient increases in DG (Rittenhouse-Simmons, 1979), IP_2 and IP_3 (Agranoff *et al.*, 1983; Watson *et al.*, 1984). In thrombin-stimulated platelets, a brief acidification is followed by alkalinization, as a result of the activation of platelet membrane Na^+/H^+ exchange (Zavoico *et al.*, 1986). A role for elevated pH_i in Ca^{2+} mobilization has been proposed (Siffert and Akkerman, 1987;Siffert and Akkerman, 1988) but this remains a controversial issue (Rink, 1987). Thrombin also

causes a guanine nucleotide-dependent inhibition of adenylate cyclase in human platelet membranes (Aktories and Jakobs, 1984). However, decreases in cAMP play no role in platelet activation (Haslam *et al.*, 1978b).

Studies investigating the importance of the above biochemical processes in thrombin-induced platelet activation, have indicated that they contribute to but may not fully account for all platelet responses. The involvement of other mechanisms besides the guanine nucleotide-dependent activation of phospholipase C in dense granule secretion is supported by the failure of GDPβS to block DG formation and [^{3}H]5-HT secretion caused by addition of high thrombin concentrations to electropermeabilized platelets (Haslam and Davidson, 1984b) and by a study showing a lack of correlation between the inhibitory actions of GDPβS on DG formation and [^{3}H]5-HT secretion at different thrombin concentrations in saponin-permeabilized platelets (Brass *et al.*, 1986). Moreover, a difference in the role of agonist-induced Na^+/H^+ exchange in platelet activation was observed with low and high concentrations of thrombin. Inhibition of Na^+/H^+ exchange attenuates dense granule secretion induced by low but not high thrombin (Sweatt *et al.*, 1985). Since secretion by low concentrations of thrombin is dependent on the formation of AA metabolites and the release of AA from the membrane phospholipids is blocked by the removal of extracellular Na^+, Na^+/H^+ exchange is thought to facilitate the

mobilization of AA that is critical for platelet activation by weak agonists (Sweatt *et al.*, 1985). Knight *et al.* (1984) have suggested that different mechanisms are responsible for dense granule secretion and lysosomal enzyme secretion even though both of these processes have the same Ca^{2+} requirement in permeabilized human platelets. Thrombin-induced secretion of 5-HT and lysosomal enzymes can also be separated on the basis of receptor occupancy. Holmsen *et al.* (1981) found that brief and prolonged exposure to thrombin was required for 5-HT and lysosomal enzyme secretion, respectively.

Thrombin causes a change in conformation of glycoproteins IIb-IIIa on the outside of the plasma membrane. This glycoprotein complex is the receptor for fibrinogen and is essential for platelet aggregation, providing sites on platelet surfaces for fibrinogen to crosslink platelets (reviewed by Phillips *et al.*, 1988). Investigation of thrombin-induced fibrinogen receptor expression in saponin-permeabilized human platelets has demonstrated that pertussis toxin-sensitive guanine nucleotide-dependent activation of phospholipase C and the formation of TxA_2 from AA mediate exposure of the fibrinogen receptor caused by low doses of thrombin (Shattil and Brass, 1987). However, since fibrinogen receptor exposure by high concentrations of thrombin or by OAG and PMA was not inhibited by aspirin or GDPβS, another pathway was thought to be responsible for their effects.

1.6.2. *AVP*

AVP is a polypeptide hormone that is synthesized in the hypothalamus, subsequently stored in the posterior pituitary and secreted into the systemic circulation upon neural stimulation. Besides causing antidiuretic effects at nM concentrations via V_2-receptors, higher concentrations of AVP have pressor and platelet-aggregating effects (Haslam and Rosson, 1972) mediated by the V_1 type of the AVP receptor (Michell *et al.*, 1979 ;Sawyer *et al.*, 1981). Human platelets are most responsive to AVP in heparinized PRP (Haslam and Rosson, 1972), probably because Mg^{2+} ions are required for binding of AVP to its receptor (Vittet *et al.*, 1986). AVP causes human platelets to change shape, undergo primary and secondary aggregation and secrete the contents of the dense granules. These reponses are sensitive to indomethacin and CP/CPK (Haslam and Rosson, 1972).

In aspirin-treated platelets containing quin 2, AVP increases $[Ca^{2+}_i]$ mainly by increasing the movement of extracellular Ca^{2+} into the cell (Hallam *et al.*, 1984a). AVP also stimulates the formation of [^{3}H]inositol phosphates and [^{3}H]DG, as well as release and metabolism of [^{3}H]AA, in appropriately labelled platelets (Siess *et al.*, 1986). By inhibiting the formation of AA metabolites, it has been possible to demonstrate the primary action of AVP on phospholipase C (Siess *et al.*, 1986). In

permeabilized human platelets, AVP enhances [^{14}C]5-HT secretion caused by Ca^{2+} , presumably as a result of DG formation (Haslam and Davidson, 1984c). Activation of phospholipase C in the plasma membrane of a mammary tumor cell line by AVP is only observed in the presence of GTP (Guillon *et al.*, 1986) and AVP stimulates a GTPase activity that is additive with that caused by epinephrine or PGE_1 in human platelet membranes (Houslay *et al.*, 1986a). It is unclear whether the GTP-dependent activation of phospholipase C by AVP is a pertussis toxin-sensitive or insensitive process in human platelets. AVP-stimulated GTPase activity was not attenuated in pertussis toxin-treated human platelet membranes (Houslay *et al.*, 1986a) whereas preincubation with AVP decreased the [^{32}P]ADP-ribosylation of two 41 kDa proteins by 50% (Brass *et al.*, 1988). A GTP and Na^+-dependent inhibition of adenylate cyclase by AVP has been demonstrated in human platelet membranes but does not occur in intact platelets (Vanderwel *et al.*, 1983).

1.6.3. *PAF*

PAF is a biologically active phospholipid with the molecular structure, 1-alkyl-2-acetyl-*sn*-glycero-3-phosphocholine (reviewed by Snyder, 1985; see Fig. 1.3). This soluble compound was discovered by Benveniste *et al.* (1972) in the suspending medium of sensitized basophils after challenge with antigen. It is a potent platelet aggregating, hypotensive and allergic/anaphylactic agent

with a very diverse spectrum of actions. PAF is thought to play an important role in the pathogenesis of acute inflammation, endotoxic shock, asthma, acute allergic responses and arterial thrombosis (reviewed by Braquet *et al.*, 1987).

Unlike many other cellular mediators, PAF is synthesized from inactive precursors in the membrane (reviewed by Snyder, 1985 and Braquet *et al.*, 1987; see Fig. 1.4). PAF is mainly produced by the sequential action of PLA_2 and acetyl CoA: 1-alkyl-2-lyso-glycero-3-phosphocholine-acetyltransferase, which convert 1-alkyl-2-acyl-*sn*-glycero-3-phosphocholine to 1-alkyl-2-lyso-glycero-3-phosphocholine (lyso-PAF) and the latter to 1-alkyl-2-acetyl-*sn*-glycero-3-phosphocholine (PAF). Small quanities of PAF are also synthesized by the transfer of phosphocholine to 1-alkyl-2-acetyl-*sn*-glycerol by 1-alkyl-2-acetyl-*sn*-glycerol: CDP-choline cholinephosphotransferase. The half-life of PAF is very short in blood due to its hydrolysis by a water-soluble 1-alkyl-2-acetyl-*sn*-glycero-3-phosphocholine:acetylhydrolase present in the intracellular and extracellular compartments that degrades PAF to the inactive lyso-PAF. Lyso-PAF can then either be reconverted to PAF via the 1-alkyl-2-lyso-glycero-phosphocholine: acetyl CoA acetyltransferase or to 1-alkyl-2-acyl-*sn*-glycero-3-phosphocholine via insertion of a long chain fatty acid by a membrane acyl CoA: 1-alkyl-2-lyso-glycero-3-phosphocholine-acyltransferase. Cellular stimuli regulate the release of PAF from the membrane

Fig. 1.3. Molecular structure of PAF

1-O-ALKYL-2-O-ACETYL-sn-
GLYCERO -3-PHOSPHOCHOLINE

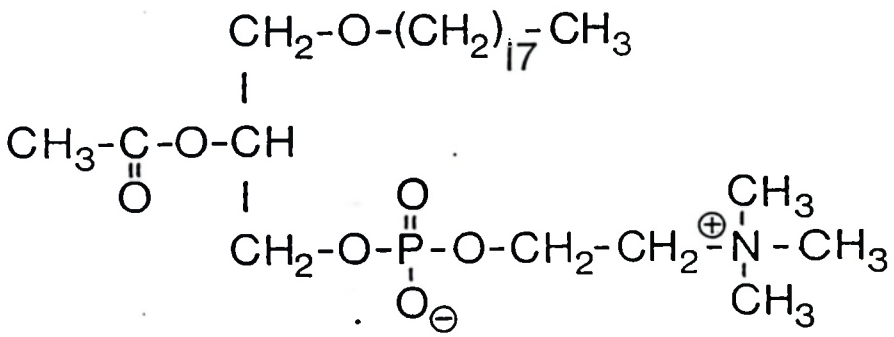
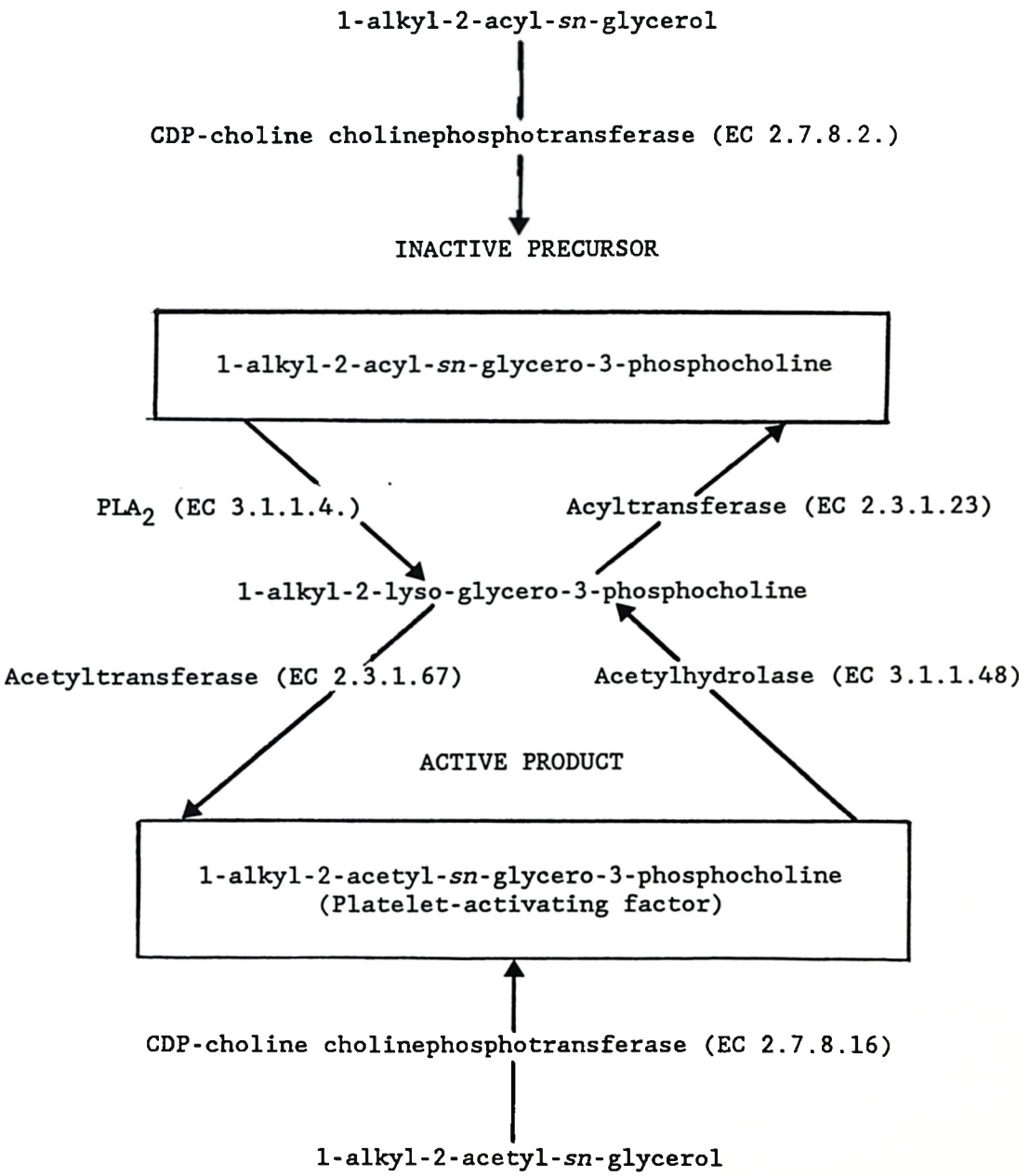


Fig. 1.4. The biosynthesis and degradation of PAF



This figure was taken from Snyder (1985).

through the Ca^{2+}-sensitive membrane-bound enzymes, PLA_2, acetyltransferase and acyltransferase. Increases in $[Ca^{2+}_i]$ favour the production of PAF by stimulating the activity of PLA_2 and acetyltransferase and by inhibiting acyltransferase (reviewed in Braquet *et al.*, 1987). Since AA is released and incorporated into membrane lipids during the cycle of deacylation-reacylation reactions involved in PAF metabolism, prostaglandin endoperoxides, TxA_2 and leukotrienes can be formed during this process and may work in concert with PAF to cause some of the above pathological effects.

The largest source of PAF is found in the blood cells and, upon activation with various stimuli, PAF is released from and subsequently activates parent and surrounding cells that contain PAF receptors. To date, PAF biosynthesis has been reported to occur in platelets, rabbit and human neutrophils, rabbit basophils, peritoneal and alveolar macrophages, eosinophils, mast cells and endothelial cells (reviewed by Braquet *et al.*, 1987). In platelets, PAF is synthesized in response to the Ca^{2+} ionophore A23187, thrombin and collagen (Chignard *et al.*, 1979; 1980). Since very little PAF is generated compared to the amounts of lyso-PAF, platelets rely on the acetylCoA-acetyltransferase activity of surrounding cells for conversion of lyso-PAF to PAF (Braquet *et al.*, 1987).

Platelets respond to PAF by changing shape, undergoing

primary and secondary aggregation and secreting the contents of the dense, α- and lysosomal enzyme-containing granules (Kloprogge *et al.*, 1983a,b). The spectrum of platelet responses observed depends on the concentration of PAF, cell type and species (Kloprogge *et al.*, 1983b; Vargaftig and Benveniste, 1983; Suquet and Leid, 1983). PAF is a very potent agonist that activates platelets in blood, plasma and physiological buffers at very low concentrations compared to most other platelet agonists (Vargaftig and Benveniste, 1983). Originally, PAF was believed to be the mediator of a 'third pathway' of aggregation that was independent of AA metabolites and released ADP and was utilized by agonists such as thrombin (reviewed in Vargaftig *et al.*, 1981). However, desensitization studies (Kloprogge *et al.*, 1983b) and the use of PAF antagonists (Adnot *et al.*, 1987) have indicated that human platelet activation by thrombin is not mediated by PAF. In addition, the contributions of AA metabolites and released ADP to platelet activation caused by PAF have been controversial and appear to be highly dependent on the platelet species investigated. For example, horse (Suqnet and Leid, 1983) and rabbit (Cazenave *et al.*, 1979; Vargaftig *et al.*, 1981) platelets respond to much lower concentrations of PAF than human platelets and the effects of secondary mediators, such as AA metabolites and ADP, are not essential for platelet activation caused by PAF in these species. However, incubation of human platelets with aspirin and CP/CPK blocks secondary aggregation and secretion caused by PAF, suggesting that shape change and primary aggregation are the

platelet responses specific to PAF in man (Chesney, 1982; Kloprogge *et al.*, 1983b; Osterman, 1983).

Investigation of signal transduction pathways utilized by PAF has shown that both increases in $[Ca^{2+}_i]$ and stimulation of phosphoinositide metabolism are involved in platelet activation by this phospholipid (Hallam and Rink, 1984b). As with other platelet agonists, most of the increase in $[Ca^{2+}_i]$ in quin-2 loaded human platelets incubated with PAF is due to the agonist-induced influx of extracellular Ca^{2+} (Hallam *et al.*, 1984b; Clare and Scrutton, 1984; Avdonin, 1987). Although increases in $[Ca^{2+}_i]$ coincide with platelet activation, the ability of PAF to cause human platelet aggregation and secretion at basal $[Ca^{2+}_i]$ (Hallam *et al.*, 1984b) and the lack of Ca^{2+} influx during primary aggregation (Clare and Scrutton, 1984) suggests that other pathways must be involved. Studies by Shukla and Hanahan (1984), using ^{32}P-labelled rabbit platelets, demonstrated that the formation of $[^{32}P]PA$ preceeds the influx of $^{45}Ca^{2+}$ caused by PAF and that increases in both Ca^{2+}_i and PA were associated with secretion of $[^{3}H]$5-HT and exhibit similar dose-response curves. In appropriately labelled indomethacin-treated rabbit platelets incubated with PAF, decreases in $[^{32}P]PIP_2$ (Mauco *et al.*, 1983) and increases in $[^{3}H]IP_3$ and $[^{3}H]IP_2$ and a later accumulation of $[^{3}H]IP$ (Shukla, 1985) were reported. Human platelets treated with cyclooxygenase inhibitors respond to PAF with increases in $[^{3}H]DG$

and [^{3}H]PA and decreases in [^{32}P]PI in [^{3}H]AA- and ^{32}P-labelled preparations, respectively (MacIntyre and Pollock, 1983; Siess *et al.*, 1984). The activation of phospholipase C and platelet shape change are not dependent on the formation of AA metabolites by PAF-induced activation of PLA_2 (Siess *et al.*, 1984). Instead, AA metabolites amplify the action of PAF by causing the formation of additional second messengers during platelet aggregation and secretion (Siess *et al.*, 1984). In electropermeabilized human platelets, PAF decreases the requirement of 5-HT secretion for Ca^{2+} (Haslam and Davidson, 1984b) and increases the formation of DG (Haslam *et al.*, 1985). PAF decreases cAMP formation caused by PGE_1 in rabbit platelets and inhibits adenylate cyclase activity in rabbit and human platelet membranes (Haslam and Vanderwel, 1982).

1.6.4. *Prostaglandin endoperoxides and TxA_2*

These biologically active eisocanoids enhance the formation of a hemostatic plug by amplifying the platelet activation caused by other agonists and by stimulating other platelets in the circulation to adhere to and aggregate at the injury site. Formation of these compounds is dependent on the liberation of AA from the *sn*-2 position of the membrane phospholipids, phosphatidylcholine, phosphatidylethanolamine and PI (Imai *et al.*, 1982). Although the exact mechanism responsible for the mobilization of AA remains to

be determined, two pathways have been identified for increasing the intracellular concentration of free AA; (1) activation of various species of PLA_2 by μM Ca^{2+}, (2) sequential degradation of DG by DG lipase and monoglyceride lipase (Prescott and Majerus, 1983). The latter appears to be a minor pathway (Mahadevappa and Holub, 1986). Studies on the effects of inhibitors of Na^+/H^+ exchange and of removal of extracellular Na^+ have suggested that intracellular alkalinization caused by agonist-induced stimulation of Na^+/H^+ exchange is critical to the mobilization of AA by weak agonists (Sweatt *et al.*, 1985). Upon release of AA, this fatty acid is rapidly converted to the prostaglandin endoperoxides, PGH_2 and PGG_2 by fatty acid cyclooxygenase, followed by metabolism to TxA_2 by thromboxane synthetase. The former enzyme is the target of non-steroidal anti-inflammatory agents such as aspirin and indomethacin (Vane, 1971). The limiting factor in the production of AA metabolites by the above sequence of reactions is thought to be the generation of free AA (Irvine, 1982). The prostaglandin endoperoxides and TxA_2 are fairly strong platelet agonists that elicit their effects in the nM range by acting on the same receptor on the platelet membrane (Rink, 1986). However, they are very unstable compounds that have short biological half-lives under physiological conditions. The half-life of TxA_2 (~30s) is much shorter than that of PGH_2 (Hamberg *et al.*, 1974 and 1975).

The actions of PGG_2, PGH_2 and TxA_2 on the signal

transduction pathways involved in platelet activation have been studied using the stable endoperoxides analogues, U44069 and U46619. Human platelets incubated with U46619 change shape, express fibrinogen receptors, aggregate and secrete the contents of the dense granules (Morinelli *et al.*, 1987). Platelet activation by stable endoperoxide analogues is associated with the receptor-mediated stimulation of phosphoinositide breakdown (Pollock *et al.*, 1984; Rittenhouse, 1984; Siess *et al.*, 1985), elevation of cytoplasmic Ca^{2+} (Pollock *et al.*, 1984) and further release and metabolism of AA. In quin-2 loaded human platelets suspended in a medium containing $^{45}Ca^{2+}$, it has been reported that the influx of extracellular Ca^{2+} through receptor-operated Ca^{2+} channels in the platelet membrane is responsible for most of the increase in $[Ca^{2+}_i]$ caused by U46619 (Avdonin *et al.*, 1987). The guanine nucleotide-dependent formation of $[^{32}P]PA$, $[^{3}H]IP_2$ and $[^{3}H]IP_3$ in saponin-permeabilized labelled human platelets incubated with stable analogues of prostaglandin endoperoxides occurs by a pertussis toxin-insensitive activation of phospholipase C (Brass *et al.*, 1987; 1988) and may thus involve a different G protein from that utilized by thrombin.

Thrombin, a very potent agonist that stimulates all the signal transduction mechanisms yet identified in platelets, including the formation of TxA_2, is not dependent on the action of this compound, at least at high concentrations (Rink and Hallam, 1984;

Siess *et al.*, 1983). In contrast, ADP, epinephrine, suboptimal doses of thrombin (Sweatt *et al.*, 1985) and collagen (Siess *et al.*, 1983) are dependent on AA metabolites for the induction of the full spectrum of platelet responses. Studies in human platelets containing quin-2 have shown that TxA_2 formation contributes to platelet activation by inducing an increase in $[Ca^{2+}_i]$ with collagen and an increase in DG in the case of ADP (Rink and Hallam, 1984). The actions of PAF (Hallam *et al.*, 1984b; Siess *et al.*, 1984) and AVP (Hallam *et al.*, 1984a; Siess *et al.*, 1986) are also enhanced by the AA metabolites, though these agonists can alone activate phospholipase C and mobilize Ca^{2+}_i. However, the activation of phospholipase C in response to epinephrine is mediated entirely by AA metabolites (Singh *et al.*, 1983). Activation of phospholipase C in intact platelets incubated with Ca^{2+} ionophore (Rittenhouse, 1984) or in saponin-permeabilized platelets incubated with IP_3 (Brass *et al.*, 1987) was also entirely due to the Ca^{2+}-induced stimulation of PLA_2 and resultant formation of AA metabolites.

1.6.5. *ADP*

This nucleotide is a physiologically important platelet agonist that has a vital role in the initiation and development of a hemostatic plug (Born, 1962). ADP is only present in plasma after release from damaged cells (red blood cells, endothelial cells) and

activated platelets. This nucleotide causes platelets to change shape, express fibrinogen receptors, aggregate and secrete the contents of their dense and α-granules but not of their lysosomes (Zucker and Nachmias, 1985). In aspirin-treated human platelets, ADP only causes shape change and primary aggregation suggesting that AA metabolites mediate ADP-induced secondary aggregation and secretion (reviewed in Zucker and Nachmias, 1985).

Investigation of the signal transduction pathways utilized by ADP has demonstrated that platelet activation by this nucleotide is associated with increases in $[Ca^{2+}_i]$. In human platelets containing quin 2, Hallam and Rink (1985a) have demonstrated that an influx of external Ca^{2+}, presumably through an ADP-receptor operated Ca^{2+} channel, accounts for the majority of Ca^{2+} mobilized upon incubation with ADP. Comparison of the Ca^{2+}_i requirements for shape change and platelet aggregation caused by a Ca^{2+} ionophore and ADP showed that sufficient Ca^{2+} is mobilized in response to ADP to mediate shape change but not platelet aggregation (Hallam and Rink, 1985a). Since, ADP can produce shape change at a $[Ca^{2+}_i]$ below that determined necessary for this response, Hallam and Rink (1985) have proposed that other mechanisms besides agonist-induced increases in $[Ca^{2+}_i]$ are involved in the primary platelet responses to ADP. However, in contrast to many other aggregating agents, the role of the second messengers, DG and IP_3 in platelet activation by ADP is presently unclear. In

platelet suspensions responsive to thrombin, ADP had no effect on PIP_2 degradation and inositol phosphate formation in intact human platelets (Fisher *et al.*, 1985) or on the sensitivity of [^{14}C]5-HT secretion to Ca^{2+} in permeabilized human platelets (Knight and Scrutton, 1985). However, Daniel *et al.* (1987) found that stimulation of aspirin-treated human platelets with ADP caused measurable increases in PA and IP_3. ADP has also been reported to induce the liberation of small amounts of AA from membrane PI by activation of a phosphoinositide-specific PLA_2 which is triggered by Na^+/H^+ exchange (Sweatt *et al.*, 1986a). ADP also appears to open Ca^{2+} channels in the plasma membrane (Sage and Rink, 1986). However, since fibrinogen receptor exposure in response to ADP occurs in human platelets that do not produce IP_3, DG, or TxA_2 or mobilize [Ca^{2+}_i], unidentified mechanisms may contribute to ADP-induced platelet activation. Although ADP causes a marked inhibition of adenylate cyclase in both intact platelets (Haslam *et al.*, 1975) and isolated platelet membranes (Cooper and Rodbell, 1979), this does not mediate platelet activation by this agonist (Haslam *et al.*, 1978b).

1.6.6. *Epinephrine*

This catecholamine is synthesized and stored in chromaffin granules in the adrenal medulla and released into the circulation in response to physical and/or emotional stress. This hormone is a

rather variable activator of human platelets in citrated PRP. However, epinephrine can cause platelets to express fibrinogen receptors, aggregate and secrete the contents of their dense and α-granules (reviewed in Zucker and Nachmias, 1985). Epinephrine also potentiates platelet responses caused by submaximal concentrations of ADP, thrombin, collagen, AVP, U46619 (Thompson *et al.*, 1986) and PAF (Vargaftig *et al.*, 1982; Fouque and Vargaftig, 1984). However, in contrast to other agonists that cause platelet activation, platelets incubated with epinephrine do not change shape (reviewed in Zucker and Nachmias, 1985). Studies with ADP-scavenging enzymes and cyclooxygenase inhibitors have shown that secondary aggregation and secretion caused by epinephrine are mediated by AA metabolites and released ADP (Siess *et al.*, 1984). Many workers argue that ADP is essential for fibrinogen receptor exposure and platelet aggregation and that the only true effect of epinephrine is to potentiate the effects of other agents (Plow and Marguerie, 1982; Figures *et al.*, 1986). Peerschke (1982) has, however, concluded that primary aggregation is mediated by epinephrine but can be potentiated by ADP. Thus, fibrinogen receptor exposure (Peerschke, 1982) and primary aggregation (Bygdeman *et al.*, 1969) may be the only specific effects of the occupation of α_2-adrenergic receptors by epinephrine.

The signal transduction mechanisms utilized by epinephrine to cause platelet activation are poorly understood. Unlike most

other agonists that stimulate platelets, epinephrine does not appear to mobilize Ca^{2+} from internal stores (Ware *et al.*, 1986) or activate PI metabolism in human platelets (Siess *et al.*, 1984; Clare and Scrutton, 1984), when the production of AA metabolites and the action of released ADP has been prevented. However, different methods of monitoring $[Ca^{2+}_i]$ have yielded opposing results and the importance of the reported influx of $^{45}Ca^{2+}$ into platelets incubated with epinephrine (Owen *et al.*, 1980; Owen and LeBreton, 1981) remains a controversial issue that has not been resolved (Clare and Scrutton, 1984). In aspirin or indomethacin-treated human platelets, intracellular quin 2 detected no increase in $[Ca^{2+}_i]$ upon incubation of platelets with epinephrine (Hallam and Rink, 1984; Rao *et al.*, 1985; Ware *et al.*, 1986; Thompson *et al.*, 1986), though aqueorin (Ware *et al.*, 1986) demonstrated an increase in $[Ca^{2+}_i]$ and chlortetracycline detected a release of Ca^{2+} from the platelet membrane (Owen and LeBreton, 1981; Sweatt *et al.*, 1986b; Thompson *et al.*, 1986). These elevated levels of Ca^{2+}_i, aggregation and secretion of ATP were only detected in epinephrine-stimulated platelets when Ca^{2+} was present in the extracellular medium, suggesting that epinephrine caused an influx of external Ca^{2+} that was critical to platelet activation but produced no release of Ca^{2+} from internal stores (Ware *et al.*, 1986). Since the indicators used have different sensitivities to Ca^{2+}_i and appear to monitor different pools of intracellular Ca^{2+} (Ware *et al.*, 1986), an action of Ca^{2+} in the platelet responses

caused by epinephrine remains a possibility.

Epinephrine does not activate phospholipase C in aspirin-treated AA-labelled human platelets, as no formation of labelled DG or PA, or decrease in labelled PI, were observed (Siess *et al.*, 1984; Clare and Scrutton, 1984). In agreement with this, is the failure of this agonist to decrease the Ca^{2+}-requirement of [^{14}C]5-HT secretion in permeabilized human platelets in the absence or presence of GTP (Knight and Scrutton, 1985). In some studies, epinephrine has appeared to cause no significant release of AA or formation of AA metabolites in aspirin-treated human platelets (Clare and Scrutton, 1984; Siess *et al.*, 1984). However, in contrast to these findings, Sweatt *et al.* (1985, 1986a,b) found that epinephrine stimulates the release and formation of small amounts of AA metabolites by activating a PI-specific PLA_2. These effects required intraplatelet alkalinization, as a consequence of an increase in Na^+/H^+ exchange, and the entry of Ca^{2+} ions. Studies by Banga *et al.* (1986) support this hypothesis and further suggest that fibrinogen binding to glycoprotein IIb/IIIa is prerequisite for the stimulation of Na^+/H^+ exchange by epinephrine. Thus, Sweatt *et al.* (1986b) suggest that epinephrine mobilizes AA at low [Ca^{2+}_i] via the synergistic activation of PLA_2 by intraplatelet alkalinization and [Ca^{2+}_i]. The small amount of AA released by epinephrine is metabolized to TxA_2 which then causes secondary aggregation and secretion of human platelets as

a result of the activation of PLC and PLA_2 (Sweatt *et al.*, 1985; 1986a,b; Banga *et al.*, 1986). The guanine nucleotide-dependent inhibition of adenylate cyclase by epinephrine (Jakobs *et al.*, 1976) does not mediate platelet activation caused by this agonist but serves only to decrease the inhibitory effects of agents that increase platelet cAMP (Haslam *et al.*, 1978a,b).

1.7. *Inhibition of platelet function by cAMP*

Both inhibitory platelet agonists, such as PGI_2, PGE_1, PGD_2 and adenosine, and compounds such as methylxanthines, papaverine and pyrimidopyrimidines block platelet function by increasing platelet cAMP (Salzman, 1972; Haslam, 1973; Haslam, 1975; Haslam *et al.*, 1978a). The intracellular concentration of cAMP is increased by these agents either by stimulation of adenylate cyclase (PGI_2 etc.) or by inhibition of cAMP phosphodiesterases that degrade cAMP to 5'-AMP (methylxanthines etc.). All the actions of cAMP in platelets are thought to be mediated by cAMP-dependent protein kinases, which dissociate into regulatory (R) and catalytic (C) subunits on binding of cAMP (Taylor, 1987).

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

In the human platelet, two such enzymes have been detected by photoaffinity labelling of their regulatory subunits with

8-azido-[^{32}P]cAMP and these correspond to the type I and II protein kinases found in other cells (Salama and Haslam, 1984). Incubation of ^{32}P-labelled platelets with PGE_1 causes the phosphorylation of at least four platelet proteins, two of which are membrane-bound (P22 and P24) and two of which are soluble (P36 and P50) (Haslam *et al.*, 1980).

Although many of the targets for the inhibitory actions of cAMP on platelet function have not yet been clearly identified, cAMP has been shown to affect the concentrations and/or actions of the second messengers involved in platelet activation. cAMP has been reported to increase the ATP-dependent uptake of Ca^{2+} into platelet membrane vesicles (from the dense tubular system) (Käser-Glanzmann *et al.*, 1977; Haslam *et al.*, 1980). It appears that phosphorylation of P22 stimulates Ca^{2+} ATPase activity (Adunyah and Dean, 1987) and this protein has now been purified and named thrombolamban (Fisher and White, 1987). In intact platelets containing quin 2, cAMP was observed to block both Ca^{2+} release from internal stores and Ca^{2+} influx caused by PAF (Hallam *et al.*, 1984; Sage and Rink, 1985) and thrombin (Sage and Rink, 1985). Agents that elevate platelet cAMP inhibit phosphoinositide hydrolysis by phospholipase C (Rittenhouse-Simmons, 1979) and thus, decrease the agonist-induced formation of DG (Rittenhouse-Simmons; 1979; Imai *et al.*, 1983) and inositol phosphates (Watson *et al.*, 1984). Although phosphorylation of receptors by

cAMP-dependent protein kinases could account for these effects, there is support for an effect on the G protein mediating phosphoinositide breakdown. Thus, PGD_2 blocks the ability of thrombin to inhibit ADP-ribosylation of the 41 kDa substrate of pertussis toxin (Halenda *et al.*, 1986). Moreover, cAMP is a less potent inhibitor of GTPγS-stimulated than of GTP-stimulated secretion from electropermeabilized platelets (Knight and Scrutton, 1985; Haslam *et al.*, 1988b), suggesting that the GTPase activity of the G protein coupling Ca^{2+} receptors to phospholipase C may be stimulated by cAMP-dependent protein kinase (Haslam *et al.*, 1988b). Another well-documented target for cAMP-dependent protein kinase is the myosin light chain kinase (Hathaway *et al.*, 1981). Phosphorylation of this enzyme inhibits its activity and consequently, decrease platelet contractility by decreasing the amount of phosphorylated myosin (Hathaway *et al.*, 1981).

1.8. *Rationale for the research on which this thesis is based*

Although many platelet aggregating agents inhibit adenylate cyclase and/or activate phospholipase C, very little was known about the regulation of or relationship between these two effector systems when the research for this thesis began in late 1981. At this time, the inhibition of platelet function by compounds that stimulate adenylate cyclase and thus increase cAMP was more clearly understood. However, the platelet was an ideal model system to study

these questions, because of the variety of receptors on its surface that couple to these effectors and the ease of preparing homogeneous platelet preparations. In addition, a recently discovered aggregating agent, PAF, had just been found to be more potent in molar terms as an inhibitor of adenylate cyclase than ADP or epinephrine in both human and rabbit platelets membranes (Haslam and Vanderwel, 1982).

Although hormonal inhibition of adenylate cyclase was known to be dependent on GTP in most cell systems (Limbird, 1981), the role of Na^+ in the platelet system in which this cation appeared itself to inhibit adenylate cyclase (Steer and Wood, 1981) is less clear. Thus, studies were undertaken to analyse the effects of NaCl and GTP on the inhibition of adenylate cyclase in which this cation appeared itself to inhibit the enzyme (Steer and Wood, 1981). Since inhibitors of Ca^{2+}-dependent proteolysis had not been generally used in the preparation of platelet particulate fractions for assay of adenylate cyclase (Steer and Wood, 1981) and proteolysis was known to attenuate hormonal inhibition of the enzyme (Stiles and Lefkowitz, 1982; Ferry *et al.*, 1982), the ability of this process to restrict the inhibition by PAF was investigated.

As incubation of platelets with excitatory agonists (Zucker and Nachmias, 1985) including PAF (Kloprogge *et al.*, 1983a,b) caused them to change shape, aggregate and secrete the contents of their granules by a process that was unrelated to decreases in cAMP

(Haslam *et al.*, 1978b), studies were underway in a number of laboratories to determine the signal transduction mechanism underlying platelet activation. Phosphoinositide breakdown was ultimately identified as the mechanism responsible for Ca^{2+} mobilization which is central to many physiological events including platelet activation (Berridge and Irvine, 1984). With the discovery of the GTP-dependence of receptor-mediated activation of phospholipase C by many of the aggregating agents that also inhibit adenylate cyclase (PAF, thrombin and AVP) (Siess *et al.*, 1984; 1986; Haslam and Davidson, 1984a,b,c), it became conceivable that similar receptors and/or G proteins were involved in these two effector systems. By modifying assay conditions used previously for measurement of adenylate cyclase and phospholipase C alone, I examined the action of PAF and thrombin on both enzymes under identical conditions using the same rabbit platelet particulate fractions. Thus, the regulatory requirements for GTP and NaCl in the inhibition of adenylate cyclase and activation of phospholipase C by PAF could be compared. This dual assay system was also used in an attempt to determine whether the G protein thought to mediate the inhibition of adenylate cyclase also coupled Ca^{2+}-mobilizing receptors to phospholipase C.

Since PAF was known to have inhibited adenylate cyclase activity in human and rabbit platelet membranes and in intact rabbit platelets (Haslam and Vanderwel, 1982), it was expected to do so in

intact human platelets. However, I found that it did not. Studies were therefore carried out to determine whether differences in assay conditions for measurement of adenylate cyclase and cAMP formation or in the environment of membranes of intact and broken platelets were responsible for this anomalous finding. During the course of these studies, activation of PKC by PMA or Ca^{2+}-mobilizing agonists was reported to alter hormonal stimulation and inhibition (See Section 1.5.4.). In platelets, PMA decreased the inhibition of adenylate cyclase by epinephrine (Jakobs *et al.*, 1985) and the active form of purified PKC was found to phosphorylate the α_i subunit of G_i (Katada *et al.*, 1985). Based on these findings, Jakobs *et al.* (1985) proposed that activation of PKC may account for the inability of some agonists that activate phospholipase C to inhibit adenylate cyclase in the intact human platelet. To test this hypothesis, I examined the relationship between activation of PKC by PMA, diC_8 and platelet agonists and the effects of these compounds on cAMP formation in intact human platelets using platelets prelabelled with both [^{3}H]adenine and [^{32}P]P_i so that [^{3}H]cAMP and incorporation of ^{32}P into platelet PKC substrates could be determined in the same samples. These experiments refuted the hypothesis of Jakobs *et al.* (1985) and suggested a hitherto unrecognized mechanism may regulate the effector on which occupied receptors act in the platelet.

Chapter 2

Experimental

2.1. *Materials*

2.1.1. *Radiolabelled compounds*

[2,8-^{3}H]ATP (27 Ci/mmol) and [2,8-^{3}H]adenine (38 Ci/mmol) were from ICN Radiochemicals (Irvine, CA, U.S.A.). [α-^{32}P]ATP (30 Ci/mmol), [8-^{14}C]cAMP (50 mCi/mmol) and carrier-free [^{32}P]P_i were obtained from NEN Canada Limited (Lachine, Que., Canada) and *myo*-[2-^{3}H]inositol (15 Ci/mmol) was from American Radiolabelled.Chemicals (St. Louis, MO, U.S.A.). One batch (1 mCi) of [2,8-^{3}H]ATP (27 Ci/mmol) was found to be contaminated with a labelled impurity that co-purified with [^{3}H]cAMP. This material was diluted with disodium ATP to a specific activity of approximately 20 mCi/mmol and then applied to a small column containing Dowex 50 resin (Bio-Rad AG 50 W-X8) and freed from the contaminating compound by elution with water. The [^{3}H]ATP obtained was adjusted to a pH of 7.4 with Tris.

[^{32}P]NAD^{+} was synthesized from [α-^{32}P]ATP and NMN by the transfer of ADP-ribose from ATP to NMN by NAD^{+} pyrophosphorylase, according to the method of Cassel and Pfeuffer (1978). NMN (10 mM), NAD^{+} pyrophosphorylase (0.1 unit/ml), $MgCl_2$ (10 mM), Mops (25 mM, buffered to pH 7.4 with NaOH) and [α-^{32}P]ATP (0.1-0.2 mM, 5-15 Ci/mmol) were mixed together

giving a final volume of 1.0 ml and incubated for 30 min at 37°C. After stopping the reaction by immersion of the mixture in a boiling water bath and removal of denatured protein, the supernatant was applied to a column (0.8cm x 4 cm) containing Dowex 1 resin (BioRad AG 1-X8, 200-400 mesh) in the formate form. [^{32}P]NAD^+ was isolated from the other compounds in the reaction mixture by elution with 0.1 M formic acid. The first 4 ml containing residual NMN was discarded and the next 6 ml, which contained 60-70% of the [^{32}P]NAD^+, was collected, lyophilized and then resuspended in 50 μl of water. The purity of the [^{32}P]NAD^+ obtained was determined by chromatography of samples of the fractions collected on polyethylenimine cellulose sheets, using 0.1 M LiCl/0.5 M formic acid as a solvent. Unlabelled NAD^+, NMN, ATP, ADP and AMP were used as standards and run in parallel with the samples.

2.1.2. *Proteins and enzymes*

CPK (150 units/mg of protein), α-chymotrypsin, CP (Tris and disodium salts), BSA (Fraction V), protein standard solution [5% (w/v) human albumin and 3% (w/v) human globulin] were purchased from Sigma Chemical Company (St. Louis, MO, U.S.A.). Human fibrinogen (Grade L) from A.B. Kabi (Stockholm, Sweden) that had been DFP-treated was a gift from Dr. J.F. Mustard (McMaster University, Hamilton). Potato apyrase was purified by ammonium sulphate fractionation of the enzyme isolated by adsorption to and elution

from calcium phosphate, according to the method of Molnar and Lorand (1961). At this stage, the precipitate was resuspended in a small volume of water, dialysed against 0.9% NaCl and stored in small aliquots in the freezer. Pertussis toxin was obtained from List Biological Laboratories, Inc. (Campbell, CA, U.S.A.) and the S_1 subunit of pertussis toxin was donated by Dr. S. Cockle of Connaught Laboratories (Toronto, Ont.).

2.1.3. *Fine chemicals and pharmacological agents*

Sigma Chemical Company (St. Louis, MO, U.S.A.) was the source for cAMP, GTP, Tris-ATP, disodium ATP (prepared by phosphorylation of adenosine and therefore essentially GTP-free), ADP, NAD^+, $NADP^+$, Tris base, Hepes, Pipes, Mops, EDTA, EGTA, indomethacin, ADP, (-)-epinephrine (+)-bitartrate, AVP, PMA, 4α-PDD, phorbol, DTT, NEM, IBMX, leupeptin, LiCl and choline chloride. Disodium ATP (GTP-free) was converted to the Tris salt by eluting the nucleotide with water from a small column containing Dowex 50 resin (Bio-Rad AG 50 W-X8) and adding Tris to pH 7.4. NaCl and KCl were of analytical grade and purchased from BDH Chemicals (Toronto, Ont., Canada). Forskolin was purchased from Behring Diagnostics (San Diego, CA, U.S.A.) and diC_8 from Avanti Polar Lipids (Birmingham, AL, U.S.A.).

The following compounds were gifts. Synthetic platelet activating factor and analogues were from Dr. H.R. Baumgartner and

Dr. R. Barner of F. Hoffman-La Roche (Basel, Switzerland) and PGE_1 was from Dr. J. Pike of the Upjohn Company (Kalamazoo, M.I.). Human α-thrombin (2700 units/mg) was provided by Dr. J.W. Fenton II of New York State Department of Health (Albany, N.Y., U.S.A.). (-)-Propranolol was from from I.C.I. (Macclesfield, U.K.).

2.1.4. *Chromatographic materials*

Neutral alumina (WN-3) was obtained from Sigma Chemical Company (St. Louis, MO, U.S.A.). Dowex 50 resin (AG 50W-X8, 200 to 400 mesh, H^+ form) and Dowex-1 anion exchange resin (AG.1-X8, 100-200 mesh, formate form) were purchased from Bio-Rad Laboratories Ltd (Mississauga, Ont., Canada). Polyethylenimine cellulose-UV 254 precoated plastic sheets were from Macherey and Nagel (Duren, Germany) and silica-gel (Si250) t.l.c. plates from J.T. Baker Chemical Company (Phillipsburg, NJ, U.S.A.).

2.1.5. *Electrophoretic materials*

SDS, acrylamide, *N,N'*-methylenebisacrylamide, *N,N,N',N'*-tetramethylethylenediamine, ammonium persulphate, bromphenol blue, 2-mercaptoethanol, Coomassie brillant blue R and the proteins used to determine the molecular weights of the ^{32}P-labelled proteins were purchased from Sigma Chemical Co. (St. Louis, MO, U.S.A.). Whatman 3MM chromatography paper was from

Whatman Chemical Separation Division (New Jersey, U.S.A.) and Cronex 4 x-ray film was obtained from E.I. Dupont de Nemours and Co. (Wilmington, U.S.A.).

2.1.6. *Liquid scintillation cocktails*

Ready-Solv HP/b scintillant obtained from Beckman Instruments (Toronto, Ont., Canada) was used for counting [^{32}P]cAMP. ^{3}H-Labelled inositol phosphates, which were isolated in large volumes of aqueous material containing high concentrations of ammonium formate, and [^{3}H]cAMP were counted in ACS scintillant from Amersham Canada Ltd. (Oakville, Ont., Canada). A 0.01% aqueous solution of 4-methylumbelliferone (Sigma Chemical Co., St. Louis, MO, U.S.A.) was used to count ^{32}P incorporated into platelet proteins on dried gels as Čerenkov radiation.

2.2. *Methods*

2.2.1. *Collection of blood and preparation of PRP*

Human blood was obtained with informed consent from healthy volunteers who claimed not to have ingested any medication during the previous 10 days. Blood was taken from the forearm by venipuncture and usually collected into a siliconized flask containing 0.175 volumes of ACD anticoagulant (85 mM trisodium citrate, 71.4 mM citric

acid, 2% (w/v) glucose) to give a final pH of 6.5 (Aster and Jandl, 1964). In some experiments, 99 volumes of whole blood was mixed with 1 volume of 0.154 NaCl containing 1000 units of heparin/ml.

Blood was also taken from rabbits anesthetized by an intravenous injection of a 6.5% solution of sodium pentobarbital in 0.154 M NaCl (30 mg/kg). Blood was withdrawn from a cannula attached to the carotid artery into plastic syringes containing 0.175 volumes of ACD anticoagulant.

To obtain PRP, whole blood was centrifuged twice at 160 $g_{av.}$ for 15 min at room temperature. PRP was removed from the buffy coat and red cells with a siliconized Pasteur pipette after each centrifugation, collected into siliconized conical tubes, and stored at 37°C. When preparing heparinized PRP, whole blood was centrifuged at 37°C.

2.2.2. *Labelling of washed human platelets with [^{3}H]adenine*

Washed human platelets were prepared by a modification of the method of Mustard *et al.* (1972). To isolate the platelets, PRP prepared from whole blood as in Section 2.2.1. was centrifuged at 1700 $g_{av.}$ for 10 min at 37°C. The platelet pellet was resuspended in 40 ml of Tyrode's solution (137 mM NaCl, 2.7 mM KCl, 11.9 mM $NaHCO_3$, 0.42 mM NaH_2PO_4, 1 mM $MgCl_2$, 2 mM $CaCl_2$,

5.6 mM glucose) supplemented with 5 mM Hepes (adjusted to pH 7.35 with NaOH), 0.35% BSA, 60 μg of apyrase/ml and 50 units of heparin/ml and was then incubated for 20 min at 37°C. During this incubation period, a platelet count was obtained by counting a fraction of the platelet suspension in a hemocytometer chamber by phase contrast microscopy (Harker, 1974) (see Section 2.2.16.). The platelet suspension was then centrifuged at 1100 $g_{av.}$ for 10 min at 37°C, and the pellet (2 to 4 x 10^{10} platelets) was resuspended in 20 ml of a medium identical to that above except that it contained no heparin. After incubation with 2 μM [^{3}H]adenine (10 Ci/mmol) for 90 min at 37°C, the platelets were then centrifuged as before and finally resuspended in a Tyrode's solution containing 5 mM Hepes (pH 7.35), 0.35% BSA and only 6 μg of apyrase/ml to give a final platelet concentration of 4 to 5 x 10^8 platelets/ml. Addition of this low concentration of apyrase prevents stimulation of the platelets by traces of ADP that may be released during storage of the suspension, but does not block the immediate effects of addition of higher (micromolar) concentrations of ADP. However, when 30 to 60 μg of apyrase/ml are added to more concentrated platelet suspensions (2.5 to 5 x 10^9 platelets/ml), platelet aggregation caused by 10 μM ADP was blocked.

2.2.3. *Labelling of washed human platelets with both [^{3}H]adenine and [^{32}P]P_i*

Platelets were labelled with both [^{3}H]adenine and [^{32}P]P_i by a modification of the method described in Section 2.2.2. The platelets (2 to 4 x 10^{10}) were first incubated for 30 min at 37°C with 2 μM [^{3}H]adenine in 10 ml of phosphate-free Tyrode's solution (137 mM NaCl, 2.7 mM KCl, 11.9 mM $NaHCO_3$, 1 mM $MgCl_2$, 2 mM $CaCl_2$ and 5.6 mM glucose) supplemented with Hepes buffer, BSA and apyrase as described in Section 2.2.2. After centrifugation of the platelets and their resuspension in 8 ml of the same phosphate-free medium, they were incubated for 60 min at 37°C with 2 mCi of carrier-free [^{32}P]P_i. The platelets were then centrifuged, washed once more in medium containing phosphate and finally resuspended in albumin-free Tyrode's solution containing phosphate, Hepes buffer and 6 μg of apyrase/ml to give a final platelet concentration of 3 to 5 x 10^8 platelets/ml. The platelet suspension was kept in a stoppered siliconized flask at 37°C until it was used.

2.2.4. *Labelling of washed rabbit platelets with [^{3}H]adenine*

Washed rabbit platelets were prepared by a modification of the method of Ardlie *et al.* (1970). PRP obtained from whole blood as in Section 2.2.1. was centrifuged at 2000 $g_{av.}$ for 15

min at room temperature and the platelet pellets were then resuspended in a Ca^{2+}-free Tyrode's solution (137 mM NaCl, 2.7 mM KCl, 11.9 mM $NaHCO_3$, 0.42 mM NaH_2PO_4, 2 mM $MgCl_2$, 5.6 mM glucose) supplemented with 0.2 mM EGTA, 0.35% BSA and 5 mM Pipes (pH adjusted to 6.5 with NaOH). After a 15 min at room temperature during which a platelet count was obtained, the platelet suspension was centrifuged at 1400 $g_{av.}$ for 10 min at room temperature. The pellet was resuspended at 2.5 x 10^9 platelets/ml in a second Ca^{2+}-free Tyrode's solution containing the same compounds as above except for the omission of EGTA. This platelet suspension was incubated with 2 μM [^{3}H]adenine (10 Ci/mmol) for 90 min at room temperature to ensure adequate uptake of [^{3}H]adenine (60-80%) and then centrifuged. The platelets were finally resuspended in a Tyrode's solution containing 5 mM Hepes, pH 7.4 and 6 μg of apyrase/ml to give a final platelet concentration of 3 to 5 x 10^8 platelets/ml. The platelet suspension was warmed to 37°C and kept in a stoppered siliconized flask until it was used.

2.2.5. *Labelling of washed rabbit platelets with [^{3}H]inositol*

Washed rabbit platelets were prepared as described in Section 2.2.4. with the following modifications to facilitate labelling with [^{3}H]inositol in place of [^{3}H]adenine (Hrbolich *et al.*, 1987). The platelet pellets (5 to 8 x 10^{10} platelets) were resuspended at 5 x 10^9 platelets/ml in a similar

Ca^{2+}-free Tyrode's solution but with the addition of 10 mM rather than 5 mM Pipes, pH 6.5, to buffer the lactic acid formed on prolonged incubation at this higher platelet concentration. This platelet suspension was incubated with 25 μCi of [^{3}H]inositol/ml for 2 h at 37^{o}C and then centrifuged and washed with the above Ca^{2+}-free Tyrode's solution to remove residual [^{3}H]inositol. All washing solutions were supplemented with 30 μg of apyrase/ml to block platelet activation by released ADP.

2.2.6. *Preparation of washed human and rabbit platelets for isolation of particulate fractions used in the assay of adenylate cyclase*

PRP was centrifuged at 2000 $g_{av.}$ for 10 min to isolate rabbit platelets and at 1700 $g_{av.}$ for 10 min to isolate human platelets. The platelet pellets were removed from residual red cells by resuspension and centrifugation three times in a 'citrate-dextrose' washing solution (13 mM sodium citrate, 5 mM glucose, 135 mM NaCl; adjusted to pH 6.5 with HCl) (Haslam and Lynham, 1972). These suspensions were centrifuged in an angle rotor at 400 $g_{av.}$ for 10 min at 4^{o}C. The final platelet pellet, essentially free of red and white cells, was weighed and then resuspended at 25 mg wet weight/ml in 150 mM Tris-HCl (pH 7.4) containing either no other additions, 5 mM EGTA or 400 μM leupeptin. Approximately 500 mg wet weight of platelets was obtained

from 250 ml of human blood and about 400 mg from the blood of one rabbit.

2.2.7. *Preparation of particulate fractions from human and rabbit platelets for measurement of adenylate cyclase*

Washed human and rabbit platelet suspensions prepared as described in Section 2.2.6. were rapidly frozen in a solid CO_2/acetone mixture and stored for up to 4 days at -50°C, before thawing in a 37°C water bath with shaking to maintain the temperature of the platelet material at 0°C. The resulting lysates were centrifuged at 37,500 $g_{av.}$ for 40 min at 4°C to give pellets containing about 40% of the total platelet protein. In many experiments, these particulate fractions were resuspended, using a Dounce homogenizer (A pestle), in a medium containing all the components of the adenylate cyclase assay mixture except [^{3}H]ATP and the compounds or salts under investigation. However, when the action of added GTP on platelet adenylate cyclase was studied, endogenous guanine nucleotides were removed by homogenization of the pellets in a hypotonic buffer containing 10 mM Tris-HCl and 5 mM Tris-EGTA, pH 7.4. After centrifugation as before and removal of the supernatant, this procedure was repeated. The platelet particulate fraction was then isolated by a final centrifugation and resuspended for assay.

2.2.8. *Assay of adenylate cyclase activity*

About 30 min after resuspension of the platelet particulate fraction, 150 μl (containing 100-200 μg of membrane protein) was mixed with 80 μl of other additions, the mixture was incubated for 1 min at 30°C to permit temperature equilibration and the assay was then started by addition of 20 μl of 5 mM Tris-[^{3}H]ATP (2 μCi). Final assay mixtures (250 μl) all contained 75 mM Tris-HCl (pH 7.4), 5 mM $MgCl_2$, 0.4 mM [^{3}H]ATP, 4 mM Tris-CP, 20 units of CPK/ml, 1 mM cAMP, 1 mM IBMX, 1 mg of crystallized BSA/ml, 0.4 mM DTT and 0.4 mM Tris-EGTA. GTP-free ATP that had been converted to the Tris salt (Section 2.1.3.) was used in experiments in which the effects of GTP were studied; otherwise commercial Tris-ATP was used. After incubation for 10 min at 30°C, assays were terminated by addition of 0.5 ml of ice-cold 15% (w/v) TCA ; samples were left on ice for about 1 h before removal of precipitated protein by centrifugation at 15,000 g for 2 min. All assays were carried out in triplicate.

2.2.9. *Preparation of rabbit platelet particulate fraction for measurement of both adenylate cyclase and phospholipase C activities*

Washed rabbit platelets prepared as described in section 2.2.5. were finally resuspended at 2 x 10^9 platelets/ml in a medium

(pH adjusted to 7.4 with NaOH) containing 100 mM NaCl, 25 mM Hepes, 2.5 mM EGTA, 1.23 mM ATP, and 1.56 mM $MgCl_2$. They were then frozen and thawed twice (see section 2.2.7.) before centrifugation of the lysate at 37,500 $g_{av.}$ for 40 min at 4°C. The platelet particulate fraction was washed twice with a medium (pH adjusted to 7.4 with NaOH) containing 25 mM Hepes, 2.5 mM EGTA, 1.23 mM ATP and 1.56 mM $MgCl_2$. The presence of MgATP was necessary to preserve membrane polyphosphoinositides during these procedures (Hrbolich *et al.*, 1987). After the second wash, the platelet pellet was finally resuspended at a membrane protein concentration of about 1 mg/ml (equivalent to 3 x 10^9 platelets/ml) in a solution (pH 7.4) containing 25 mM Hepes, 2.5 mM EGTA, 100 mM NaCl, 0.96 mM $MgCl_2$, 0.46 mM ATP and 1.67 mg of BSA/ml .

2.2.10. *Measurement of adenylate cyclase and phospholipase C activities in particulate fraction prepared from the same rabbit platelets*

Incubation mixtures contained 150 μl of membrane suspension from platelets labelled with [^{3}H]inositol (100-200 μg of membrane protein) and 100 μl of other additions dissolved in a medium (pH adjusted to 7.4 with NaOH) containing 100 mM NaCl, 25 mM Hepes and 2.5 mM EGTA. The final concentrations of compounds used in these assays were; 100 mM NaCl, 25 mM Hepes, 2.5 mM EGTA, 0.5 mM Mg^{2+}_{free}, 0.4 mM MgATP, 1 mg of BSA/ml, 1.48 mM

$CaCl_2$ (final pCa of 7 at pH 7.4), 4 mM CP, 20 units of CPK/ml, 0.4 mM DTT and 1 mM IBMX. [α-^{32}P]ATP (28 Ci/mmol) was added only to those assay tubes in which adenylate cyclase activity was determined. Reactions were initiated by simultaneous addition of 150 μl of labelled membranes, 25 μl of IBMX and 12.5 μl of $CaCl_2$/KOH to the other reagents which were pre-pipetted into the assay tubes. By adding 29.6 mM $CaCl_2$/80 mM KOH to each sample, a final $CaCl_2$ concentration of 1.48 mM and pCa of 7 was obtained (calculated according to Fabiato and Fabiato, 1979). Potassium hydroxide was required to neutralize the $2H^+$ released from EGTA by Ca^{2+}. After incubation of the samples for 10 min at 25°C, reactions were stopped by addition of 0.125 ml of ice-cold 30% (w/v) TCA. Because of the difficulty of purifying the ^{3}H-labelled inositol phosphates in the presence of ^{32}P-labelled adenine nucleotides, separate assay tubes were used to measure the phospholipase C and adenylate cyclase activities of the membranes, though the incubation conditions were identical.

2.2.11. *Isolation of labelled cAMP and calculation of adenylate cyclase activities*

Labelled cAMP was isolated from adenylate cyclase assay mixtures by the method of Jakobs *et al.* (1976), as modified by Haslam and McClenaghan (1981). To monitor the recovery of labelled cAMP during this procedure, 1000 d.p.m. of [^{14}C]cAMP (50 mCi/mmol)

was added to each acidified incubation mixture. After centrifuging to remove protein, the supernatants were added to columns (15 cm x 0.7 cm) containing 1.5 g of alumina (WN-3) that had just been acidified with 10% (w/v) TCA. Each column was then washed with 9 ml of TCA, 9 ml water and 2 ml of 0.2 M ammonium formate (pH 6.0), after which 3 ml of 0.2 M ammonium formate was added to elute the labelled cAMP. The eluate was then applied to a column containing 1.5 ml (packed volume) of Dowex 50 resin (Bio-Rad AG 50W-X8, 100-200 mesh, H^+ form) followed by 6 ml of 1 mM potassium phosphate buffer, pH 7.35. Cyclic AMP was then eluted with a further 9 ml of the potassium phosphate buffer, which was lyophilized overnight and counted in a mixture of 8 ml of ACS or Ready-solv HP/b scintillant and 0.5 ml of water. In experiments in which the [^{3}H]cAMP formed by platelet particulate fractions was measured, each sample was counted for ^{3}H and ^{14}C in ACS scintillant, using a Beckman LS 230 scintillation counter with an efficiency of about 15% for ^{3}H and 45% for ^{14}C. However, when [^{32}P]cAMP formation was measured in simultaneous assays of adenylate cyclase and phospholipase C, lyophilized samples were counted for ^{32}P and ^{14}C in Ready-solv HP/b scintillant with an efficiency of almost 100% for ^{32}P and about 52% for ^{14}C.

In order to determine the d.p.m. of [^{3}H]cAMP formed in adenylate cyclase assays, the counts were corrected for background radioactivity, crossover of ^{14}C into the ^{3}H channel and

variations in quenching. The d.p.m. of ^{3}H found in samples incubated in the absence of platelet membranes or in unincubated samples (100-200 d.p.m.) were subtracted to give values that were then corrected for the recovery of [^{14}C]cAMP. The total d.p.m. of [^{3}H]cAMP formed were then expressed as nmoles/10 min/mg of membrane protein after division by the specific activity of the [^{3}H]ATP added and the membrane protein in each sample. All these assays were carried out in triplicate.

Values for [^{32}P]cAMP formation were calculated similarly, except that it was necessary to correct the results for decay of ^{32}P after subtraction of the background count. Determination of the recovery of [^{14}C]cAMP required correction for both background and crossover of ^{32}P (6-7% of the counts in the ^{32}P channel). Blank values for [^{32}P]cAMP obtained in samples with no membranes or without incubation were very low (30-50 c.p.m.). [^{3}H]Inositol phosphates present in [^{32}P]cAMP samples were not detected in either the ^{32}P or ^{14}C channels. These assays were carried out in triplicate except for those containing membranes treated with pertussis holotoxin, which were performed in duplicate.

2.2.12. *Isolation and measurement [^{3}H]inositol phosphates*

Acidified samples identical to those used to monitor adenylate cyclase activity (apart for the ommission of

[α-^{32}P]ATP), were centrifuged to remove platelet protein and the supernatants were neutralized with NaOH, using bromthymol blue as a indicator. After dilution to 5 ml with water and thorough mixing, each sample was applied to a column containing 1.25 ml of Dowex 1 anion exchange resin (AG1-X8: 100-200 mesh, formate form) for the subsequent separation of the [^{3}H]inositol phosphates. The stepwise elution method used to isolate inositol phosphates was a modification (Hrbolich *et al.*, 1987) of the method of Berridge *et al.* (1983) designed to prevent contamination of the IP fraction with some IP_2. Inositol was eluted with 3 x 5 ml of distilled water and glycerophosphoinositol with 2 x 5 ml of 5 mM disodium tetraborate/60 mM sodium formate. IP, IP_2 and IP_3 were then eluted with 3 x 5 ml 0.15 M ammonium formate in 0.1 M formic acid, 4 x 5 ml 0.4 M ammonium formate in 0.1 M formic acid and 3 x 5 ml 1.0 ammonium formate in 0.1 M formic acid, respectively. Each of the 5 ml fractions was then adjusted to 1.0 M ammonium formate, so that the final salt concentrations were the same in samples containing IP, IP_2 and IP_3 fractions. This prevented phase separation when the samples were mixed with 15 ml of ACS scintillation fluid. The samples were counted for ^{3}H for 8 min in a LS 3801 liquid Beckman scintillation counter. To determine the formation of inositol phosphates, the following calculations were carried out. The c.p.m. in the ^{3}H channel was first corrected for background radioactivity and quenching (efficiency of counting ^{3}H was 14%) and the d.p.m. present in fractions containing each inositol phosphate were summed.

The values from triplicate assays were averaged and the average d.p.m. of each inositol phosphate present in unincubated controls was then subtracted. The results (means ± S.E.D.) were then expressed as d.p.m. of IP, IP_2 or IP_3 formed/10 min per mg of membrane protein.

2.2.13. *[^{32}P]ADP-ribosylation of pertussis toxin substrates in platelets*

Activation of pertussis toxin. Lyophilized pertussis toxin purchased from List Biologicals was reconstituted with water to give 0.5 mg of toxin/ml in 0.05 M sodium phosphate buffer, pH 7.0, containing 0.25 M NaCl. This material was incubated with 2 M urea for 4 h at 4°C prior to an experiment to dissociate the toxin subunits, which was followed by activation for 60 min at 30°C with 0.1 mM ATP and 20 mM DTT. S_1 subunit of pertussis toxin (from Connaught Laboratories) was stored at 0.4 mg/ml in 2 M urea, 0.1 M NaCl, 0.1 M potassium phosphate buffer, pH 7.0 and 0.1 mM ATP and activated before use by incubation for 60 min at 30°C with 0.1 mM ATP and 20 mM DTT. Activated holotoxin was diluted 8 to 20-fold and activated S_1 subunit 3-fold into incubation mixtures containing platelet membranes and the reagents necessary for ADP-ribosylation of G proteins.

[^{32}P]ADP-ribosylation of platelet particulate fraction by

pertussis toxin. Platelet lysate was centrifuged at 37,500 $g_{av.}$ for 40 min at 4°C and the particulate fraction was resuspended at about 4 mg of membrane protein/ml in a solution containing 1 mM ATP (disodium salt), 0.1 mM GTP, 10 mM thymidine, 10 mM arginine, 5 mM CP (disodium salt), 16 units of CPK/ml, 1 mM EDTA, 1 mM EGTA, 1 mM $NADP^+$, 1 mM NAD^+, 5 mM $MgCl_2$ and 25 mM Hepes, pH 7.7. This suspension was then divided into two parts, one of which was incubated for 30 min at 37°C with 50 μg of activated pertussis toxin/ml and the other of which was used as a control and therefore incubated under the same conditions with the same reagents, but in the absence of pertussis toxin. These incubations were terminated by an 8 to 10-fold dilution of the platelet suspension with a solution (pH adjusted to 7.4 with NaOH) containing 25 mM Hepes, 2.5 mM EGTA, 1.23 mM ATP and 1.56 mM $MgCl_2$ (buffer A), followed by immediate centrifugation at 37,500 $g_{av.}$ for 40 min. The pellets were resuspended in buffer A, recentrifuged again and finally resuspended for assay of phopholipase C and adenylate cyclase activities, as described for experiments without pertussis treatment.

Measurement of the extent of [^{32}P]ADP-ribosylation of platelet polypeptides by pertussis toxin. Samples of control and pertussis toxin-treated membrane suspensions (0.1 ml) were removed just before the ADP-ribosylation reaction was terminated and the enzyme activity in these preparations was stopped by dilution with 1 ml of buffer A and centrifugation at 37,500 $g_{av.}$ for 40 min.

The resulting platelet pellets were washed a second time with the same volume of buffer A. After further centrifugation, both pellets were resuspended in the ADP-ribosylation incubation mixture described above (final volume 0.1 ml) but with the replacement of 1 mM NAD^+ by 10 μM [^{32}P]NAD^+ (1-2 Ci/mmol). These mixtures were incubated for 90 min at 30°C with 50 μg of activated pertussis toxin/ml. The reactions were stopped by dilution with buffer A and centrifugation, and the resulting pellets were dissolved in 50 μl of electrophoresis sample buffer (Laemmli, 1970) and then heated at 100°C for 3 min. Equal amounts of protein from the control and pertussis toxin-treated suspensions (approx. 50-80 μg) were analyzed by SDS/polyacrylamide-slab gel electrophoresis using 13% (w/v) acrylamide in the separating gel. Labelled polypeptides were located by autoradiography and those in the 38-42 kDa range were cut from dried gels and placed in polythene vials containing 10 ml of 0.01% (w/v) 4-methylumbelliferone in water. These samples were then counted for Čerenkov radiation (Haslam *et al.*, 1979a) with an efficiency of about 60%. Background radioactivity (about 30 c.p.m.) and decay of ^{32}P were taken into account in the quantification of the amount of ^{32}P present. The molecular masses assigned to ^{32}P-labelled proteins were determined by interpolation using the commercial standards listed in Section 2.2.15. The extent of ADP-ribosylation of platelet substrates by incubation with pertussis toxin and unlabelled NAD^+ was calculated from the ratio of ^{32}P incorporated into the test and control membranes during the second

ADP-ribosylation reaction.

2.2.14. *Measurement of [^{3}H]cAMP formation in intact human and rabbit platelets*

Experiments were carried out about 30 min after final resuspension of platelets that had been isolated, washed and labelled, as described in Sections 2.2.2. to 2.2.4.. Usually, samples of washed platelet suspension (420 μl) containing 4.7 x 10^8 platelets/ml that had been prelabelled with [^{3}H]adenine or with both [^{3}H]adenine and [^{32}P]P_i were incubated for 10 min at 37°C with 20 μl of 0.154 M NaCl containing 250 μM indomethacin (to prevent prostaglandin endoperoxide and TxA_2 formation) and 20 μl of 0.154 M NaCl containing 100 mM CP and 5000 units of CPK/ml (to convert traces of ADP to the ADP receptor antagonist, ATP). Reactions were then started by the simultaneous addition of 10 μl of 0.154 M NaCl containing any activators and/or inhibitors of adenylate cyclase and 40 μl of 0.154 M NaCl containing 12.5 mM IBMX (to block cAMP phosphodiesterase activity). The total incubation volume was 500 μl and contained 10 μM indomethacin, 2 mM CP, 100 units of CPK/ml and 1 mM IBMX, as well as any other additions. Incubations (at 37°C) were stopped, usually 0.5 min later, by addition of 1 ml of 15% (w/v) TCA. However, when the protein pellets were analysed by gel electrophoresis, 1 ml of 7.5% (w/v) TCA was used.

The chromatographic isolation and measurement of [^{3}H]cAMP, using [^{14}C]cAMP to monitor the recovery of [^{3}H]cAMP has been described in Section 2.2.11. In the dual-labelling experiments designed to correlate the incorporation of ^{32}P into platelet proteins with the formation of [^{3}H]cAMP upon exposure to various stimuli, scintillation vials containing labelled cAMP were counted for 10 min in a LS 3801 Beckman scintillation counter using channel settings that permitted measurement of ^{32}P, ^{3}H and ^{14}C. The counting efficiency of ^{3}H was about 17% and that of ^{14}C about 52%. Due to the unexpected finding that cAMP from ^{32}P-labelled platelets contained ^{32}P, presumably due to significant labelling of platelet ATP in the α position, it was necessary to correct for the crossover of ^{32}P into the ^{14}C channel, in addition to the other corrections made when counting ^{14}C and ^{3}H described in Section 2.2.11.

In all these experiments, the [^{3}H]cAMP was finally expressed as a percentage of the total ^{3}H in the platelets present in the experimental samples. This was determined by counting the ^{3}H in the samples of platelet suspension and of suspending medium (obtained by centrifugation) before and after the incubations. About 95% of the ^{3}H in platelet suspensions was intracellular.

2.2.15. *Measurement of protein phosphorylation in intact platelets*

The protein precipitated by TCA (final concentration 5%) from experimental samples containing ^{32}P-labelled platelets was dissolved in electrophoresis sample buffer (Laemmli, 1970), which contained 3% (w/v) SDS, 0.0025% (w/v) bromphenol blue, 62 mM Tris-HCl, pH 6.8, 6% (w/v) glycerol and 5% (v/v) 2-mercaptoethanol, as described by Haslam *et al.* (1979). After neutralization of residual acid with NaOH, the samples were heated at 100°C for 3 min to ensure denaturation and reduction of the platelet proteins. Equal amounts of protein from the experimental samples (about 50 μg) were analysed by discontinuous SDS/polyacrylamide-gel electrophoresis by the method of Laemmli (1970). Polymerization of the separating gels (18 cm long) was initiated by adding a final concentration of 0.075% (w/v) ammonium persulphate to a solution containing 13% (w/v) acrylamide, 0.35% (w/v) *N,N'*-methylenebisacrylamide, 0.1% (w/v) SDS, 0.025% (v/v) *N,N,N,N'*-tetramethylethylenediamine, 0.1% (w/v) glycerol and 0.375 M Tris/HCl, pH 8.8. Stacking gels (2 cm long) placed directly above the separating gel, were polymerized similarly by the addition of 0.3% (w/v) ammonium persulphate to a solution containing 5% (w/v) acrylamide, 0.13% (w/v) *N,N'*-methylene-bisacrylamide, 0.1% (w/v) SDS, 0.038% (v/v) *N,N,N',N'*-tetramethyl-enediamine, 0.05% (w/v) glycerol and 0.125 M Tris/HCl, pH 6.8. The electrode buffer, pH 8.3, contained 0.025 M Tris, 0.192 M glycine and

0.1% SDS. Electrophoresis of the protein samples was carried out at 90 V and was continued overnight for approx. 17 h. Gels were stained for about 1.5 h with 0.1% (w/v) Coomassie Brillant Blue R in methanol/acetic acid/water (5:1:5 by vol.) and destained by gently shaking the gels for 30 min in three changes of a solution containing methanol/acetic acid/water (5:1:5 by vol.). Next, gels were placed in 10% acetic acid for 30 min and then washed thoroughly with water (2 x 30 min). Gels were dried under vacuum on Whatman 3MM chromatography paper and placed against Cronex 4 x-ray film in a cassette containing a Lightning-Plus intensifying screen (Dupont de Nemours, Wilmington, Delaware) for 3-5 days. The autoradiographs were developed in a Kodak RP X-Omat processor. Labelled polypeptides, located by autoradiography, were cut from the dried gels and counted as described in Section 2.2.13. In order to assign molecular masses to ^{32}P-labelled proteins, 10 μg each of high and low molecular weight standard mixtures containing the following purified proteins were electrophoresed in parallel with the protein samples: myosin (205 kDa), β galactosidase (116 kDa), phosphorylase b (97.4 kDa), bovine albumin (66 kDa), ovalbumin (45 kDa), glyceraldehyde-3-phosphate dehydrogenase (36 kDa), carbonic anhydrase (29 kDa), trypsinogen (24 kDa), trypsin inhibitor (20.1 kDa) and α-lactalbumin (14.2 kDa). The molecular masses of the ^{32}P-labelled polypeptides studied were either determined from the linear regression of -log R_f against the molecular masses of the above protein standards (Neville, 1971) or from from previously

assigned values for platelet phosphoproteins (Haslam and Lynham, 1977; Haslam *et al.*, 1979a).

2.2.16. *Determination of platelet protein and of the concentrations of platelets in suspensions*

Protein was quantified by the method of Lowry *et al.* (1951) using a solution of 5% (w/v) human albumin and 3% (w/v) human globulin as the protein standard. In experiments with platelet particulate fractions, 200 μl of suspension was mixed with 400 μl of 15% TCA and placed on ice for 30 min to precipitate the protein. After centrifugation, the protein pellet was resuspended in 200 μl of 1 M NaOH. After the protein had dissolved, 10, 20 and 50 μl samples were removed and brought to a final volume of 100 μl with 1 M NaOH. Each sample was then mixed with 1 ml of a solution containing 2% Na_2CO_3 in 0.1 N NaOH, 1% sodium potassium tartrate and 0.5 % $CuSO_4.5H_2O$ in the ratio 100:1:1 and incubated for 15 min at room temperature. Next, 0.1 ml of Folin reagent (1 M) was added to the samples, which were rapidly mixed and incubated for 30 min at 37^{o}C. After cooling for 30 min, sample absorbance at 500 nm was determined. Reagent blanks were subtracted and platelet protein determined from a standard curve. The albumin in the medium used to resuspend the platelet particulate fractions was also assayed and subtracted from the protein value obtained for each sample.

Platelets were counted in hemocytometer chamber by phase contrast microscopy (Harker, 1974). After mixing 10 μl of washed platelet suspension with 990 μl of 0.154 M NaCl containing 1.5% formaldehyde and 5 mM EDTA, a fraction was placed in the chamber. After sufficient time for the platelets to settle had elapsed (usually 10 min), squares containing approx. 400 platelets were counted and the platelet count was calculated. By measuring both the initial platelet count and membrane protein concentration in each experiment, it was found that 3×10^9 rabbit platelets generated about 1 mg of platelet membrane protein.

2.2.17. *Measurement of platelet aggregation*

Platelet aggregation was monitored turbidometrically using a Payton aggregometer (Scarborough, Ont., Canada). Heparinized PRP (see Section 2.2.1.) or washed platelets (3 to 4×10^8 platelets/ml) prepared as described in Section 2.2.2. were maintained at 37°C in siliconized glass tubes. Samples (final volume of 1 ml) were incubated with indomethacin for 10 min to prevent the formation of prostaglandin endoperoxides. The platelets were stirred at 1000 r.p.m. with nickel-plated round-edged stir-bars for 2 min prior to the addition of aggregating agents and incubations were stopped after a further 0.5 min by removal of the platelets from the aggregometer. When the effects of aggregating agents on washed platelets were investigated, the platelets were incubated with 0.2 mg of human

fibrinogen/ml for 0.5 min prior to the addition of the aggregating agent. The changes in light transmittance 0.5 min after addition of various concentrations of aggregating agent were determined and the concentrations causing 50% of the maximum increase in light transmittance calculated (EC_{50} value).

2.2.18. *Statistics*

Incubations were performed in triplicate in each experiment. Most tables and figures are derived from single representative experiments and give mean values ± S.E. of the mean (M), difference (D) or quotient (Q), as appropriate. Adenylate cyclase activities were expressed as mean values ± S.E.M.

$$\text{S.E.M. } (\bar{x}) = [\lesssim (\bar{x}-x)^2/n-1]^{1/2} \; / \; n^{1/2}$$

The mean zero min incubation value in the phospholipase C assay was subtracted from the mean values for [^{3}H]IP_2 formation after 10 min. Similarly, in experiments in which the formation of [^{3}H]cAMP caused by PGE_1 was measured in intact platelets, mean basal [^{3}H]cAMP values were subtracted from the mean values after stimulation by PGE_1. In these cases, the differences were expressed as means ± S.E.D.

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

In individual experiments, the effects of test additions were also expressed as percentages of the corresponding control values or as percentage changes (stimulation or inhibition) and mean percentages ± S.E.Q. are given.

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

In the above instances, the significance of changes in individual experiments was evaluated by two-sided unpaired *t* tests.

$$t_{n_T + n_C - 2} = \bar{x}_T - \bar{x}_C / [s^2(1/n_T + 1/n_C)]^{1/2}$$

$t_{n_T + n_C - 2}$ = *t* distribution with $n_T + n_C - 2$ df

$\bar{x}_T - \bar{x}_C$ = difference in independent sample means for treatment and control groups

$s^2 = [\sum (x_T - \bar{x}_T)^2 + \sum (x_C - \bar{x}_C)^2]/n_T + n_C - 2$

n_T = number of treatment samples

n_C = number of control samples

$(x_T - \bar{x}_T)^2$ = sum of squares about the mean of the treatment group

$(x_C - \bar{x}_C)^2$ = sum of squares about the mean of the control group

The effects described were obtained in at least three separate experiments, and statistical information indicating the variation encountered between experiments is given in the text, usually as mean values ± S.E.M. The significance of differences

between treatments observed in multiple experiments were evaluated by two-sided paired *t* tests.

$$t_{n-1} = \bar{d}\text{-}0 \,/[s_d/(n)^{1/2}] \qquad s_d = [\,(d-\bar{d})^2/n\text{-}1]^{1/2}$$

t_{n-1} = *t* distribution with n-1 df

n = number of paired observations

d = difference for each of n paired observations

$\bar{d}$ = mean of sample differences

s_d = sample standard deviation of the differences

When the results clearly did not exhibit a normal distribution, the significance of the difference between paired samples in multiple experiments was evaluated by the Wilcoxon signed rank test. The test statistic was calculated in the following way. The differences beteen paired samples were ranked ignoring their sign and then reassigned positive or negative signs according to the sign of the original difference. Then the sum of the positive or negative signs were evaluated for significance at p = 0.05

One-way ANOVA was used to determine if decreases in PGE_1-stimulated cAMP formation by sub-maximal doses of epinephrine could be significantly enhanced by addition of 40 nM or 400 nM PAF (Chapter 5).

Chapter 3

Regulation of Rabbit and Human Platelet Adenylate Cyclase by Na^+, GTP and PAF

3.1 *Introduction*

3.1.1. *Inhibition of adenylate cyclase by platelet aggregating agents*

The blood platelet is widely used as a model system for investigation of the receptor-mediated inhibition of adenylate cyclase (Jakobs *et al.*, 1981; Cooper, 1982; Limbird, 1983). Two inducers of platelet aggregation, epinephrine and ADP, have long been known to inhibit the accelerated rates of cAMP formation observed in intact platelets exposed to PGE_1 (for review see Haslam *et al.*, 1978). Both agonists have also been shown to inhibit the adenylate cyclase activity of isolated platelet membrane preparations via specific receptors (Jakobs *et al.*, 1976; Cooper and Rodbell, 1979). However, a third aggregating agent, PAF, is on a molar basis a much more potent inhibitor of adenylate cyclase, both in intact rabbit platelets and in particulate fractions from this species (Haslam and Vanderwel, 1982). The structural requirements for this effect were highly specific and, although inhibition of enzyme activity was also observed with human platelet particulate fractions, none was detected with rabbit liver membranes (Haslam and Vanderwel, 1982). Thus, it is likely that specific membrane receptors mediate this action of PAF.

3.1.2. *Regulation of hormone-mediated inhibition of adenylate cyclase by GTP and NaCl*

Hormone-mediated inhibition of adenylate cyclase, has been shown to depend on the presence of GTP (reviewed by Gilman, 1987). In many biological systems, NaCl potentiates hormonal inhibition of adenylate cyclase by stimulating basal enzyme activity in the presence of GTP, an effect that is reversed by the inhibitory hormone (Jakobs *et al.*, 1981; Cooper, 1982; Limbird, 1983). In the platelet, the inhibitory effect of epinephrine requires GTP (Jakobs *et al.*, 1978; Steer and Wood, 1979) but the role of Na^+ is less clear. Thus, NaCl has been reported to inhibit the adenylate cyclase activity of membrane preparations from human platelets, an effect that was merely additive with GTP-dependent inhibition of activity by epinephrine (Steer and Wood, 1981; Mooney *et al.*, 1982). On the other hand, Michel *et al.* (1980) found that NaCl potentiated the inhibitory action of epinephrine on the PGE_1-stimulated adenylate cyclase activity of rabbit platelet particulate fraction.

3.1.3. *Effects of proteolysis on platelet adenylate cyclase*

Treatment of a variety of cells and membrane preparations with proteolytic enzymes has been reported to enhance basal adenylate cyclase activity and to modify the regulation of this enzyme by hormones and guanine nucleotides (Hanoune *et al.*, 1982). In the

case of the rat platelet, incubation of homogenate with $CaCl_2$ caused a 2 to 7-fold increase in enzyme activity that could be blocked by leupeptin or NEM, indicating that this effect was attributable to the action of an endogenous Ca^{2+}-dependent thiol protease (Adnot *et al.*, 1982). In addition, experiments with human platelets have demonstrated that exogenous serine proteases such as trypsin block the inhibition of PGE_1-stimulated (Stiles and Lefkowitz, 1982) and basal (Ferry *et al.*, 1982) adenylate cyclase activities by epinephrine. These effects were attributed to the loss of the function of the G_i protein that couples inhibitory receptors to the catalytic moiety of adenylate cyclase (Stiles and Lefkowitz, 1982; Ferry *et al.*, 1982). In contrast, receptor-mediated activation of the enzyme was unimpaired by proteolysis (Stiles and Lefkowitz, 1982).

3.1.4. *Objectives of this study*

The objectives of the first part of this thesis were therefore to explore the roles of NaCl and GTP in regulating rabbit and human platelet adenylate cyclase activity in the absence and presence of PAF. In studies with particulate fractions prepared from platelet lysates resuspended in Tris buffer, the maximal inhibitions by PAF of basal activity were variable and relatively low (20-50%) (Haslam and Vanderwel, 1982). Since limited proteolysis can suppress the effects of inhibitory hormones on adenylate cyclase, another

objective of this study was to investigate the possibility that endogenous proteolysis restricted the inhibition caused by PAF. This investigation was carried out using rabbit and human platelet particulate fractions prepared in the both the absence and presence of proteolytic inhibitors.

3.2. *Results*

3.2.1. *Effects of NaCl and PAF on the basal adenylate cyclase activities of rabbit and human platelet particulate fractions*

When platelets were lysed in the presence of EGTA, the adenylate cyclase activity of the particulate fraction was stimulated by NaCl (Fig. 3.1.). With material from both rabbit and human platelets, 40 mM NaCl caused approx. 2-fold increases in enzyme activity. However, the extent and pattern of stimulation by higher NaCl concentrations was different in the two species. With rabbit platelet particulate fraction, enzyme activity increased linearly between 40 and 200 mM NaCl, reaching about 8-fold higher than the control at the latter concentration (Fig. 3.1.). In contrast, the increase in enzyme activity with human platelet particulate fraction was roughly hyperbolic, reaching a maximum of 2.5-fold at 200 mM NaCl (Fig. 3.1.). In the presence of 100 mM NaCl, which was used in most experiments, the increase in adenylate cyclase activity amounted

to 5.6 ± 0.4-fold and 2.2 ± 0.1-fold in rabbit and human platelet particulate fractions (means ± S.E.M.; from 11 and 4 expts., respectively).

Addition of 100 nM PAF inhibited the adenylate cyclase activities of rabbit and human platelet particulate fractions to similar extents in the absence of NaCl (35 ± 2% and 39 ± 2%; means ± S.E.M. from 8 and 3 expts., respectively). In the case of the rabbit material, 100 nM PAF also prevented the increases in adenylate cyclase activity attributable to NaCl (Fig. 3.1.), with the result that the inhibition of total enzyme activity reached a maximum of 90 ± 1% (mean ± S.E.M., 6 expts.) in the presence of 100 mM NaCl. With the human enzyme, PAF almost completely blocked the effects of NaCl concentrations up to 100 mM, at which a maximum inhibition of 64 ± 3% (mean ± S.E., 4 expts.) was achieved (Fig. 3.1.).

The effectiveness of different concentrations of PAF in inhibiting platelet adenylate cyclase was studied using both rabbit and human platelet particulate fractions (Fig. 3.2.). With both preparations, increasing concentrations of agonist overcame the stimulatory effects of 40 mM, 100 mM and 200 mM NaCl on adenylate cyclase activity, but the higher concentrations of NaCl caused shifts to the right in the dose-response curves. For example, in this experiment, addition of 200 mM NaCl increased the concentration of PAF causing half-maximal inhibition from 0.6 to 2.3 nM in rabbit

platelet particulate fraction and from 0.7 to 7.4 nM with human material. The ability of different concentrations of NaCl to increase the concentration of PAF required for half-maximal inhibition did not correlate with the stimulation of basal adenylate cyclase activity by this salt. Thus, NaCl increased rabbit platelet adenylate cyclase activity more than it did human, but caused a larger shift in the dose-response curves with enzyme from the latter species. Moreover, NaCl concentrations above 100 mM had little effect on human platelet adenylate cyclase activity but caused marked shifts in the dose-response curve (Fig. 3.2.). Addition of choline chloride (up to 200 mM) had no effect on the dose-response curves for inhibition of human platelet adenylate cyclase by PAF in either the presence or absence of NaCl (Table 3.1.). Since the stimulatory action of NaCl did not correlate with its ability to shift the dose-response curve for PAF to the right, these two effects of NaCl may be mediated through different sites.

3.2.2. *Role of GTP in the actions of NaCl and PAF on human and rabbit platelet adenylate cyclase activity*

To demonstrate effects of GTP on inhibition of adenylate cyclase by PAF, it was first necessary to remove endogenous guanine nucleotides from the particulate fractions used. It was found that after two washes of the human platelet material with a hypotonic buffer (10 mM Tris-HCl + 5 mM Tris-EGTA, pH 7.4), inhibition of

enzyme activity by PAF and stimulation by NaCl was completely dependent on added GTP (Fig. 3.3.). Comparison of the effects of different concentrations of GTP showed that this nucleotide had biphasic effects on enzyme activity, tending to stimulate at low concentrations, an effect that was potentiated by NaCl, and to inhibit at higher concentrations, an effect that was diminished by NaCl. Optimal stimulation was observed with about 0.1 μM GTP in the absence of NaCl and with 2 μM GTP in the presence of 100 mM NaCl (Fig. 3.3., A). As a result, the increase in enzyme activity caused by NaCl grew progressively larger with 0.02-2 μM GTP. However, the inhibition of adenylate cyclase activity by PAF was unaffected by NaCl at GTP concentrations $\leq$ 0.4 μM and was optimal with 10 μM GTP (Fig. 3.3., B). Thus, the effects of NaCl on basal enzyme activity and on the inhibitory action of PAF were expressed over different ranges of GTP concentration, suggesting that they may be mediated by distinct G proteins with different affinities for GTP.

With rabbit material, only a partial dependence of inhibition on GTP could be demonstrated after two hypotonic washes were carried out as described in Section 2.2.7. Addition of 10 μM GTP to rabbit platelet particulate fraction that had been washed with hypotonic buffer inhibited adenylate cyclase activity by 40% in the absence of NaCl but stimulated the enzyme by about 60% in the presence of 150 mM NaCl (Table 3.2.). As a result, the increase in

basal enzyme activity caused by NaCl was considerably enhanced by added GTP. Inhibition of the rabbit platelet enzyme by PAF was unaffected by added GTP in the absence of NaCl (about 20% inhibition) but was markedly enhanced by GTP in the presence of 150 mM NaCl (from about 40% to 90%).

3.2.3. *Effects of proteolysis on rabbit platelet adenylate cyclase activity*

The basal adenylate cyclase of particulate fractions from rabbit platelets lysed in the absence of EGTA was about 5 times higher than the activity of fractions prepared in the presence of EGTA, although the assays were carried out under identical conditions (see Fig. 3.4., A and B). NaCl had opposite effects on the activities of these two preparations. Addition of 10-150 mM NaCl inhibited the activity of the preparation from which EGTA was omitted (maximum 45% at 50 mM) (Fig. 3.4., A), though as already shown in Fig. 3.1., it stimulated that of preparations from platelets lysed in the presence of EGTA by up to 10-fold (Fig. 3.4., B). In the former preparation, NaCl often had a biphasic effect, causing substantially less inhibition in the presence of 150 mM NaCl than 50 mM NaCl (Fig. 3.4., A). Addition of EGTA to the platelet lysis buffer and inclusion of NaCl in the assay medium both resulted in an enhanced inhibition of rabbit platelet adenylate cyclase by 100 nM PAF (Fig. 3.4., D). This was mostly attributable to blockade of the

NaCl-stimulated adenylate cyclase activity by PAF. Thus, 150 mM NaCl increased the inhibition caused by 100 nM PAF from 8 ± 2 to 67 ± 3% (mean values ± S.E.M., 4 expts.) when EGTA was ommitted, and from 24 ± 1 to 88 ± 1% (mean values ± S.E.M., 4 expts.) when EGTA was used. The effects of use of EGTA on both basal adenylate cyclase activity and the inhibitory action of PAF suggested that significant Ca^{2+}-dependent proteolysis occurred when platelets were lysed in the absence of this compound.

The actions of EGTA were compared with those of leupeptin to confirm that the former compound acted by inhibiting proteolysis. Addition of increasing concentrations of NaCl in the absence and presence of 100 nM PAF to platelets lysed in the presence of 400 μM leupeptin produced the same qualitative changes in adenylate cyclase activity as observed in particulate fractions that were prepared from platelets lysed in EGTA. Thus, addition of 100 nM PAF blocked the stimulation of adenylate cyclase by NaCl seen in particulate fractions prepared with leupeptin, with the result that inhibition of the enzyme increased from 20-30% in the absence of NaCl to about 80% in the presence of 150 mM NaCl (Fig. 3.4., C). The concentration of NaCl required for half-maximal inhibition of adenylate cyclase by PAF was markedly increased by proteolysis (Fig. 3.4., D).

Incubation of rabbit platelet lysate containing 5 mM EGTA

with 50 μg of α-chymotrypsin/ml prior to isolation of the particulate fraction resulted in an up to 6-fold stimulation of basal adenylate cyclase activity (Fig 3.5., A and C). Under these conditions, NaCl caused a more pronounced inhibition of the enzyme than observed after endogenous proteolysis, probably because the stimulatory action of this salt was completely or almost completely lost. A biphasic effect of NaCl, similar to that usually observed after endogenous proteolysis, was obtained when a lower α-chymotrypsin concentration was used (Fig. 3.5., B). Pre-incubation of lysate with α-chymotrypsin diminished the inhibition of adenylate cyclase activity by PAF in a dose-dependent manner (Fig. 3.5., D); with 50 μg of protease/ml, no inhibitory effect of PAF was detected in the absence of NaCl and only a very weak inhibition was observed with 150 mM NaCl.

3.2.4. *Effects of proteolysis on the role of GTP in regulating the actions of NaCl and PAF on rabbit platelet adenylate cyclase activity*

Incubation of platelet lysate containing 5 mM EGTA for 15 min at 25°C with 5.2 mM $CaCl_2$ to give a final [Ca^{2+}] of 200 μM, reduced both the percentage inhibition of adenylate cyclase activity by 10 μM GTP observed in the absence of NaCl and the percentage stimulation caused by GTP in the presence of 150 mM NaCl (Table 3.2.). Enhancement of endogenous Ca^{2+}-dependent proteolysis by

addition of $CaCl_2$ to platelet lysates, also decreased the stimulation of adenylate cyclase activity by NaCl in both the presence and absence of GTP. Although proteolysis substantially reduced the inhibition of adenylate cyclase by PAF in the presence of GTP, no difference was observed between proteolysed and non-proteolysed preparations in the absence of GTP. These studies demonstrate that the GTP dependence of the stimulatory effect of NaCl and of the inhibitory action of PAF on platelet adenylate cyclase were largely abolished by proteolysis.

3.2.5. *Specificity of the actions of NaCl on basal adenylate cyclase activity and the inhibitory action of PAF*

The actions of NaCl on rabbit platelet adenylate cyclase in particulate fractions that were prepared in the absence and presence of EGTA were compared with those of the chloride salts of other monovalent cations. With non-proteolysed platelet particulate fractions, LiCl was stimulatory, although less so than NaCl, and KCl weakly inhibitory (Table 3.3.). However, with proteolysed preparations (Table 3.3.), both LiCl and KCl were inhibitory. Choline-Cl had no effect on either preparation (Table 3.3.). These results show that neither the stimulatory nor the inhibitory action of NaCl was attributable to an increase in the ionic strength of the medium or to Cl^- ions. The effectiveness of these salts in enhancing the inhibition of adenylate cyclase by PAF in

non-proteolysed preparations decreased in the order NaCl > LiCl > KCl (Table 3.3.). Choline chloride showed no activity. The effects of these salts on the inhibition of adenylate cyclase could not be fully accounted for by the ability of PAF to block salt enhancement of enzyme activity. Thus, lower adenylate cyclase activities were observed in the presence of PAF and NaCl than with PAF alone. This effect was also seen in the presence of LiCl and KCl (Table 3.3.).

3.3. *Discussion*

3.3.1. *Effects of NaCl on the adenylate cyclase activities of non-proteolysed and proteolysed platelet particulate fractions*

The observed effects of NaCl differ markedly from those obtained in previous studies with platelet adenylate cyclase. With both human and especially rabbit platelet particulate fractions, a marked stimulation of basal adenylate cyclase activity by NaCl was found that was dependent on or potentiated by GTP. In contrast, others (Steer and Wood, 1981; Mooney *et al.*, 1982) have reported that NaCl caused a GTP-independent inhibition of the basal enzyme activity of platelet membrane preparations. Although inhibition of adenylate cyclase by NaCl has also been observed with membranes from neuroblastoma x glioma cells (Koski *et al.*, 1982), activation has been a more frequent observation with enzyme from a variety of cell

types (Jakobs *et al.*, 1979; Cooper *et al.*, 1980; Aktories *et al.*, 1981; Katz *et al.*, 1981). It has been suggested that stimulation is encountered with crude but not washed membrane preparations and is a non-specific salt effect (Koski *et al.*, 1982). However, no effect of washing platelet particulate fractions on the stimulation of adenylate cyclase by NaCl was observed in the present study, provided GTP was added (compare Fig. 3.1. and Table 3.3). Moreover, this action of Na^+ was quite specific when compared with other monovalent cations. These results agree well with those of others who have observed a moderately specific and GTP-dependent activation of adenylate cyclase by Na^+ in adipocyte ghosts (Aktories *et al.*, 1981; Katz *et al.*, 1981).

The present studies on endogenous Ca^{2+}-dependent proteolysis have shown that inhibition of adenylate cyclase by NaCl is only seen with particulate fractions from platelets lysed in the absence of 5 mM EGTA and is prevented by lysis of the platelets in the presence of leupeptin, an inhibitor of Ca^{2+}-dependent proteolysis. Therefore, it is likely that inhibition of platelet adenylate cyclase by NaCl is a proteolytic artifact and stimulation of the enzyme is the more natural response to NaCl. It may be possible to use this inhibitory effect of NaCl on adenylate cyclase as an indicator of proteolytic membrane alteration in a variety of biological systems.

Omission of EGTA from the platelet lysis buffer also had two other major effects on the adenylate cyclase activities of human and rabbit platelet particulate fractions; basal activity was increased and the inhibitory effect of PAF was diminished. Several lines of evidence indicated that these changes in enzyme activity also resulted from Ca^{2+}-dependent proteolysis of the platelet membrane. Thus, two forms of Ca^{2+}-dependent protease have been identified in platelets (Tsujinaka *et al.*, 1982) and leupeptin, a potent inhibitor of this enzyme (Toyo-Oka *et al.*, 1978), had the same effect as chelation of platelet Ca^{2+} by EGTA. Moreover, incubation of the platelet lysate with α-chymotrypsin in the presence of EGTA led to essentially the same results as omission of EGTA. The results of Adnot *et al.* (1982), who found that incubation of platelet lysate with added $CaCl_2$ enhanced adenylate cyclase activity, are in accord with this conclusion.

These studies show the importance of eliminating Ca^{2+}-dependent proteolysis during and after lysis of platelets in obtaining an optimal hormonal inhibition of particulate fraction adenylate cyclase. They also show, for the first time, that NaCl stimulates the activity of the platelet enzyme, provided proteolysis is prevented, and enhances the inhibitory effect of PAF. Under optimal conditions, this agonist caused up to 68 and 90% inhibitions of the adenylate cyclase of human and rabbit preparations, respectively.

3.3.2. *Effects of GTP on the adenylate cyclase activities of non-proteolysed and proteolysed platelet particulate fractions: modulation by NaCl*

The importance of chelating Ca^{2+} ions during lysis of platelets in order to obtain inhibition of adenylate cyclase by high GTP concentrations has been recognized previously (Tsai and Lefkowitz, 1979b). In the present studies, GTP had distinct biphasic effects on the adenylate cyclase activity of washed particulate fraction from human platelets. In the absence of NaCl, low concentrations of GTP were weakly stimulatory and higher concentrations were inhibitory. Previous reports (Steer and Wood, 1979; Tsai and Lefkowitz, 1979b) have described inhibition but not stimulation of platelet adenylate cyclase by GTP in the absence of a stimulatory hormone. However, biphasic effects of GTP have been observed in several other tissues (Cooper, 1982; Cooper *et al.*, 1980; Cooper *et al.*, 1979; Londos *et al.*, 1981) and with the platelet enzyme on addition of PGE_1 (Steer and Wood, 1979). In the presence of NaCl, the stimulatory effect of GTP was enhanced and the inhibitory phase markedly reduced. This has also been observed with membranes from rat adipocytes (Londos *et al.*, 1981) and cerebral cortex (Cooper *et al.*, 1980). These biphasic effects are readily interpreted in terms of the different GTP requirements of the G_s and G_i proteins that couple stimulatory and inhibitory receptors to adenylate cyclase (Cooper, 1982). Although the effects of a full

range of GTP concentrations was not investigated with washed particulate fraction from rabbit platelets because of the difficulty of completely removing endogenous guanine nucleotides, comparable results were obtained on addition of 10 μM GTP. Endogenous Ca^{2+}-dependent proteolysis attenuated the effects of GTP on the stimulation of adenylate cyclase activity by NaCl in rabbit platelet particulate fractions.

Several studies have shown that limited proteolysis can selectively eliminate the function of the G_i protein (Stiles and Lefkowitz, 1982; Ferry *et al.*, 1982; Aktories *et al.*, 1982b). The results of this study are in agreement with these findings since the inhibitory action of PAF on rabbit platelet adenylate cyclase, was GTP-dependent and largely abolished by proteolysis. In contrast, the stimulatory action of PGE_1 was unaffected (Williams *et al.*, 1984; Stiles and Lefkowitz, 1982).

The stimulatory and inhibitory effects of NaCl on platelet adenylate cyclase activity that have been observed, together with their different cation specificities, suggest that NaCl may act at two different sites. This conclusion maybe consistent with evidence that NaCl inhibits the effects of both the G_i and G_s GTP-binding proteins in adipocyte membranes (Aktories *et al.*, 1982b). Because inhibition by NaCl was only observed after proteolysis, when G_i-mediated effects were absent or reduced (Stiles and Lefkowitz,

1982), it is possible that this action of NaCl is attributable to inhibition of G_s function. Conversely, the stimulatory effect of NaCl could be exerted through G_i, as suggested by Aktories *et al.* (1982b). However, this hypothesis does not explain why stimulation of basal adenylate cyclase activity by NaCl requires a lower GTP concentration than the reversal of this effect by PAF, which is presumably mediated by G_i.

3.3.3. *Inhibition of adenylate cyclase activity by PAF in non-proteolysed and proteolysed platelet particulate fractions: regulation by NaCl and GTP*

The maximum inhibition of adenylate cyclase by PAF in non-proteolysed rabbit platelet particulate fraction (90%) was larger than has generally been observed with hormonal inhibitors of adenylate cyclase (Cooper, 1982). However, with human preparations the maximum inhibition (64%) was comparable with that caused by epinephrine (Jakobs *et al.*, 1976). This difference in the effectiveness of PAF in the two species is readily explained. Thus, the inhibitory effect consisted of two major components, firstly, an inhibition of the basal activity seen in the absence of NaCl, which was similar in the human and rabbit platelet particulate fractions and, secondly, a suppression of the stimulatory effect of NaCl. The overall contribution of this latter effect was greater in rabbit preparations because of the larger increases in enzyme activity

caused by NaCl. LiCl was found to potentiate inhibition of adenylate cyclase by the same mechanism in rabbit preparations. These results are in marked contrast with those obtained by others working on human platelet adenylate cyclase (Steer and Wood, 1981; Mooney *et al.*, 1982), who found no effect of NaCl on the fractional inhibition of enzyme activity by epinephrine. This can largely be explained by the failure of these workers to obtain activation of adenylate cyclase by NaCl. Another group (Michel *et al.*, 1980), using rabbit material, observed that NaCl did potentiate the inhibition of PGE_1-stimulated activity by epinephrine but did not indicate whether or not NaCl increased basal activity.

Studies by Jakobs and colleagues, have demonstrated that NaCl can decrease both GTP-dependent hormonal inhibition and stimulation of adenylate cyclase activity in human platelet membranes (Jakobs *et al.*, 1984b). Although NaCl had no effects on the actions of maximal doses of GTP or of inhibitory or stimulatory agonists on the enzyme, it did shift the dose-response curves to the right, so that larger amounts of these compounds were required to produce the same response (Jakobs *et al.*, 1984b). Analysis of the kinetics of activation of adenylate cyclase upon stimulation and inhibition of the enzyme with the stable GTP analog, GTPγS, demonstrated that the rates of activation of G_s and G_i were attenuated by NaCl (Jakobs *et al.*, 1984b). Both basal and hormone-stimulated GTPase activities of these two G proteins were also reduced by NaCl (Jakobs

et al.,1984b; Koski *et al.*, 1982). However, it is less clear from their data how this antagonistic effect of NaCl is exerted. The catalytic subunit of adenylate cyclase has been ruled out, since NaCl has no inhibitory action on forskolin-stimulated enzyme activity. NaCl also exhibited the ability to shift the dose-response curves for the inhibition of adenylate cyclase activity by PAF to the right. As this phenomenon did not correlate with the activation of adenylate cyclase by NaCl either within or between species, it is possible that NaCl exerts these two actions through different sites on the platelet membrane. Several workers have detected a decreased affinity of receptors for inhibitory agonists in the presence of NaCl (Cooper, 1982), including decreased binding of epinephrine to α_2-adrenergic receptors on the platelet membrane (Michel *et al.*, 1980; Tsai and Lefkowitz, 1978; Limbird *et al.*, 1982; Motulsky and Insel, 1983). A more recent study by Hwang *et al.* (1986) has shown that NaCl also inhibits [^{3}H]PAF binding to specific receptor sites on rabbit platelet membranes and causes a shift to the right in the displacement curve for PAF. Jakobs *et al.* (1984b) have demonstrated that NaCl decreases the affinity of β-adrenoceptors for isoproterenol in both wild type S49 lymphoma cells and the G_s-deficient cyc$^-$ cells. In the latter cell type, guanine nucleotides do not regulate β-adrenoceptor binding of agonists (Ross *et al.*, 1977). Limbird *et al.* (1982) have shown that NaCl decreases the affinity of solubilized platelet α_2-adrenergic receptors for epinephrine, implying that Na^+ can modulate receptor affinity via binding sites distinct from any

associated with the GTP-binding proteins. A Na^+ site distinct from the guanine nucleotide regulatory site is also supported by studies of human platelet and chick heart adenylate cyclase activities after treatment of the membranes with NEM or α-chymotrypsin. In the NEM-treated preparations, NaCl continued to decrease agonist affinity for the α_2-adrenoceptor despite the loss of both receptor regulation by guanine nucleotides and of adenylate cyclase inhibition by hormones and GTP (McMahon and Hosey, 1983; Limbird and Speck, 1983). Since, NEM alkylates the same cysteine residue on α_i that pertussis toxin covalently modifies and thus, blocks the ADP-ribosylation of α_i by pertussis toxin (Ui and Katada, 1987), it is unlikely that this action of NaCl is mediated by G_i. Sodium ion regulation of α_2-adrenoreceptor affinity was also preserved in human platelet membranes that were unresponsive to Gpp(NH)p after incubation with α-chymotrypsin (Periyasamy and Somani, 1987). Thus, the shift in dose-response curves for PAF could reflect a mechanism of action of Na^+ ions that is independent of G proteins, whereas the activation of adenylate cyclase by NaCl could be due to an action on the G_s and/or G_i proteins, as this effect requires GTP.

Although complete dependence of the action of PAF on GTP was observed with washed particulate fraction from human platelets, only a partial dependence on GTP was detected with rabbit material. In previous studies on the inhibitory action of epinephrine on platelet adenylate cyclase, a GTP requirement was readily demonstrated with

washed human preparations (Jakobs *et al.*, 1978; Steer and Wood, 1981; Mooney *et al.*, 1982) but was not observed at all with rabbit material (Michel *et al.*, 1980). Several workers have explained the roles of GTP and NaCl in the hormonal inhibition of adenylate cyclase in terms of the ability of the former to inhibit basal enzyme activity and of the latter to reverse this effect. Thus, inhibitory hormones have often appeared to act by blocking this GTP-dependent activation of the enzyme by NaCl (Limbird, 1983; Cooper *et al.*, 1980; Aktories *et al.*, 1981). Such a mechanism could account for the Na^+-dependent part of the inhibitory action of PAF on human and rabbit platelet adenylate cyclase. Potentiation by NaCl of the inhibition of the human enzyme required a relatively high concentration of GTP (>0.4 μM). This is consistent with many reports that hormonal inhibition of adenylate cyclase mediated by G_i requires a higher GTP concentration than hormonal activation of the enzyme mediated by G_s (Cooper, 1982). However, a stimulation of basal enzyme activity by NaCl was clearly observed with 0.02-0.4 μM GTP in the present study (see Fig. 3.3., A). This suggests that NaCl has an action on platelet adenylate cyclase at low GTP concentrations that may be mediated through a different site than that which is involved in its ability to potentiate inhibition of the enzyme by PAF. A direct or indirect stimulatory effect of NaCl on G_s activity would be consistent with our observations.

In contrast, the work of Jakobs *et al.* (1984b) with human platelet membranes has suggested that NaCl may inhibit both the G_s

and G_i proteins, so slowing their activation by GTPγS. These workers argue that at concentrations of GTP required for hormonal inhibition of adenylate cyclase, GTP is itself a fairly potent inhibitor of the enzyme and inhibitory agonists do not decrease the enzyme activity much below that seen with GTP alone. Thus, it is only when Na^+ is present to block the inhibition by GTP that the inhibitory agonist causes a substantial decrease in cAMP formation. However, in the present study, NaCl did not merely block the inhibition of adenylate cyclase by GTP, so that an effect of PAF could be detected. It also stimulated the enzyme in a GTP-dependent manner that contributes significantly to the inhibitory action by PAF. In the study by Jakobs *et al.* (1984b), all assays are carried out in the presence of forskolin. This would stimulate adenylate cyclase activity to such a degree that it would prevent detection of any further increase in enzyme activity caused by NaCl. Furthermore, if NaCl only acted to decrease the action of inhibitory agonists, it is not possible to account for the fact that rabbit platelet membrane adenylate cyclase activity was lower in the presence of PAF and NaCl than with PAF alone.

Investigation of the actions of GTP, NaCl and Mg^{2+} on the inhibition of adenylate cyclase by agonists in a number of cell types including human platelets has led to still another hypothesis of the role of these compounds in regulating hormonal inhibition of the enzyme. Bockaert *et al.* (1984) have shown that the affinity of adenylate cyclase for Mg^{2+} is critical to the activity of enzyme

and that it is altered by GTP, NaCl and inhibitory agonists. These investigators have presented evidence that GTP and inhibitory agonists lower and that NaCl increases the affinity of adenylate cyclase for Mg^{2+}. Although such a mechanism could account mechanistically for some of the observations, there is no evidence that Mg^{2+} is a physiological regulator of adenylate cyclase.

Further studies are clearly required before the actions of NaCl on adenylate cyclase activity can be fully understood. However, the present work establishes for the first time that NaCl stimulates platelet adenylate cyclase and that the inhibition of this enzyme by PAF requires GTP and is potentiated by NaCl. As PAF is an exceptionally potent inhibitor of adenylate cyclase, further analysis of its actions in the platelet system may provide additional insight into the mechanisms involved in the hormonal inhibition of this enzyme.

3.4. *Summary*

3.4.1. NaCl increased basal adenylate cyclase activity and potentiated the inhibitory action of PAF on adenylate cyclase in non-proteolysed rabbit and human platelet particulate fractions, with the largest effects in the former species. Although both these effects were dependent on GTP, they were observed with different GTP concentrations, suggesting that they may be mediated by distinct G proteins.

3.4.2. The inhibition of adenylate cyclase by PAF consisted of two components; an NaCl-independent effect and a suppression of the stimulatory effect of NaCl. The greater inhibition by PAF in rabbit material was attributable to the larger stimulation of adenylate cyclase activity by NaCl in this species.

3.4.3. NaCl shifted the dose-response curves for the inhibition of adenylate cyclase by PAF to the right in non-proteolysed rabbit and human platelet particulate fractions. The largest shifts occurred with human material. As this effect of NaCl did not correlate with the GTP-dependent stimulatory action of NaCl on adenylate cyclase activity, is may be mediated by a different Na^+ site.

3.4.4. The effects of different chloride salts of monovalent cations on adenylate cyclase in non-proteolysed and proteolysed material demonstrated that the stimulatory and inhibitory actions of NaCl are relatively specific to Na^+ and are likely to be mediated by different sites.

3.4.5. GTP had biphasic effects on human adenylate cyclase; low concentrations were stimulatory and high concentrations inhibitory, probably reflecting the actions of G_s and G_i, respectively. NaCl enhanced the stimulatory phase and diminished the inhibitory phase.

3.4.6. Ca^{2+}-dependent proteolysis altered the effects of NaCl, GTP and PAF on adenylate cyclase activity. The stimulatory action of NaCl was replaced by inhibition, basal adenylate cyclase activity was increased substantially and the inhibitory actions of GTP and PAF were reduced in proteolysed material.

Fig. 3.1. Effects of different concentrations of NaCl on the adenylate cyclase activities of platelet particulate fractions and on the inhibitory action of PAF

Assays were carried out as described in Section 2.2.8. with particulate fractions prepared from both rabbit platelets (A) and human platelets (B) that were lysed in the presence of 5 mM EGTA, as described in Section 2.2.7. All assays contained 10 μM GTP. Other additions consisted of the indicated concentrations of NaCl without (○) or with (●) 100 nM PAF. Values are means ± S.E.M from triplicate determinations.

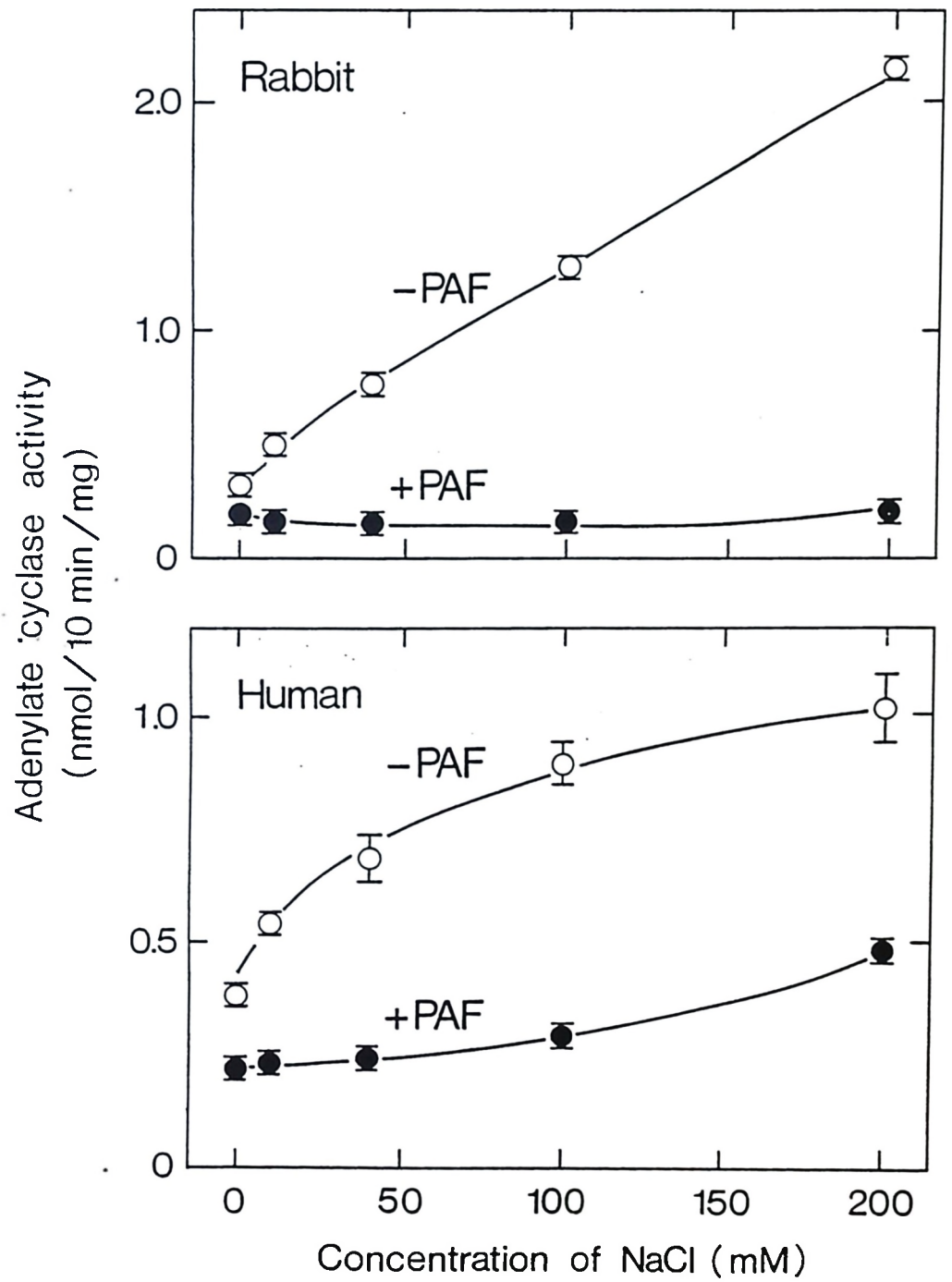

Rabbit
2.0
1.0
0
−PAF
+PAF
Human
1.0
0.5
0
−PAF
+PAF
0
50
100
150
200
Concentration of NaCl (mM)
Adenylate cyclase activity
(nmol/10 min/mg)

Fig. 3.2. Dose-response curves for the inhibition of platelet adenylate cyclase by PAF in the presence of different concentrations of NaCl

The results show the adenylate cyclase activities of particulate fractions prepared from both rabbit platelets (A) and human platelets (B) that had been lysed in the presence of 5 mM EGTA, as in Section 2.2.7. Assays contained 10 μM GTP. PAF concentrations were as indicated and the following NaCl concentrations were used: ●, none; ▼, 40 mM; ◆, 100 mM; ▲, 200 mM. Values are means ± S.E.M. from triplicate determinations.

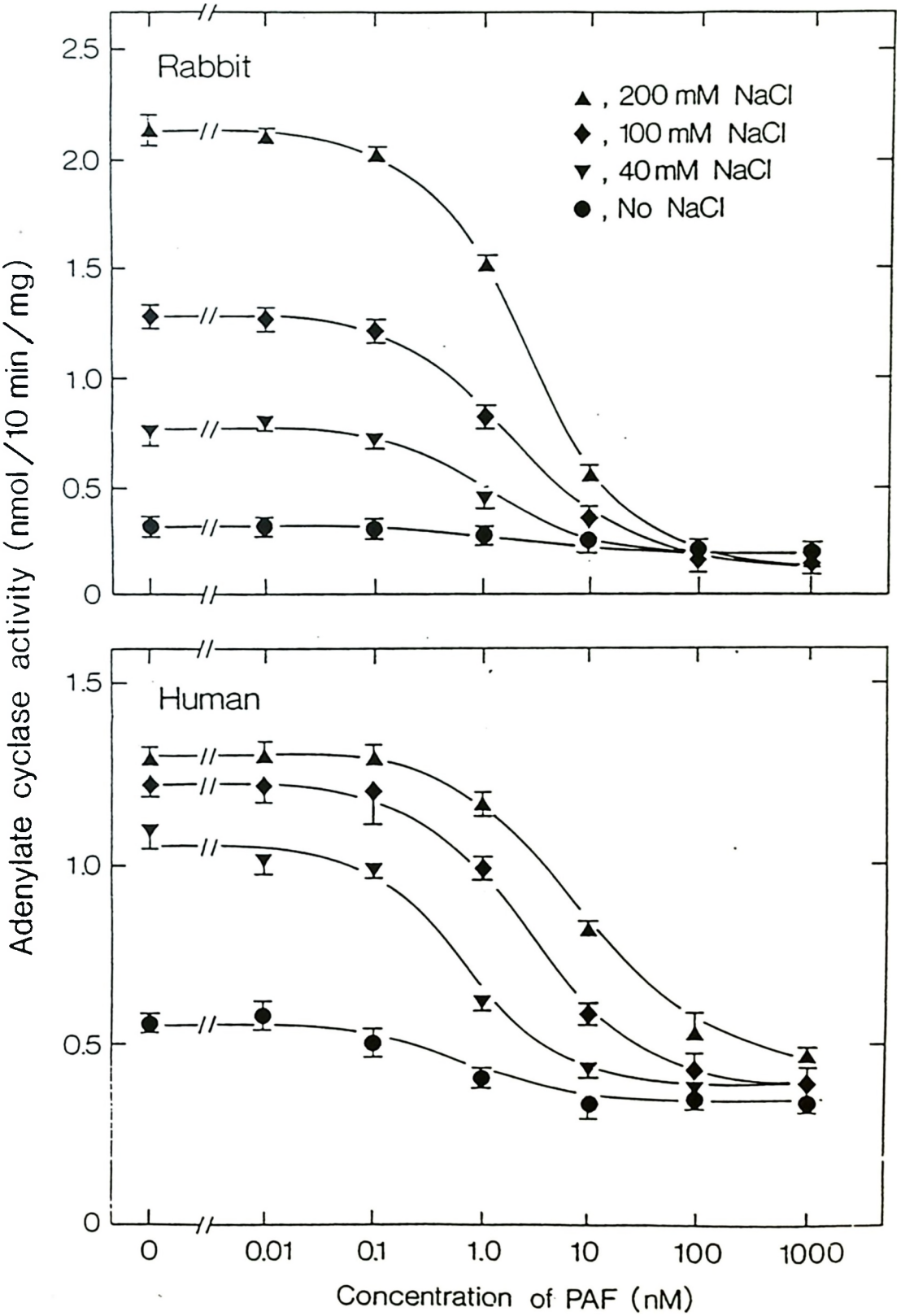

Rabbit
▲, 200 mM NaCl
◆, 100 mM NaCl
▼, 40 mM NaCl
●, No NaCl
Human
Adenylate cyclase activity (nmol / 10 min / mg)
Concentration of PAF (nM)
0
0.01
0.1
1.0
10
100
1000
0.5
1.0
1.5
2.0
2.5

Fig. 3.3. Effects of different concentrations of GTP with and without NaCl on the adenylate cyclase activity of human platelet particulate fraction; potentiation of the inhibitory action of PAF

Particulate fractions prepared from human platelets lysed in the presence of 5 mM EGTA were washed twice with hypotonic buffer prior to resuspension for assay. (A) Adenylate cyclase activities at the indicated concentrations of GTP in the absence (●) and presence (◆) of 100 mM NaCl. Values are means ± S.E.M. from triplicate determinations. (B) Inhibition of these adenylate cyclase activities by 100 nM PAF. Values are expressed as % inhibition ± S.E.Q.

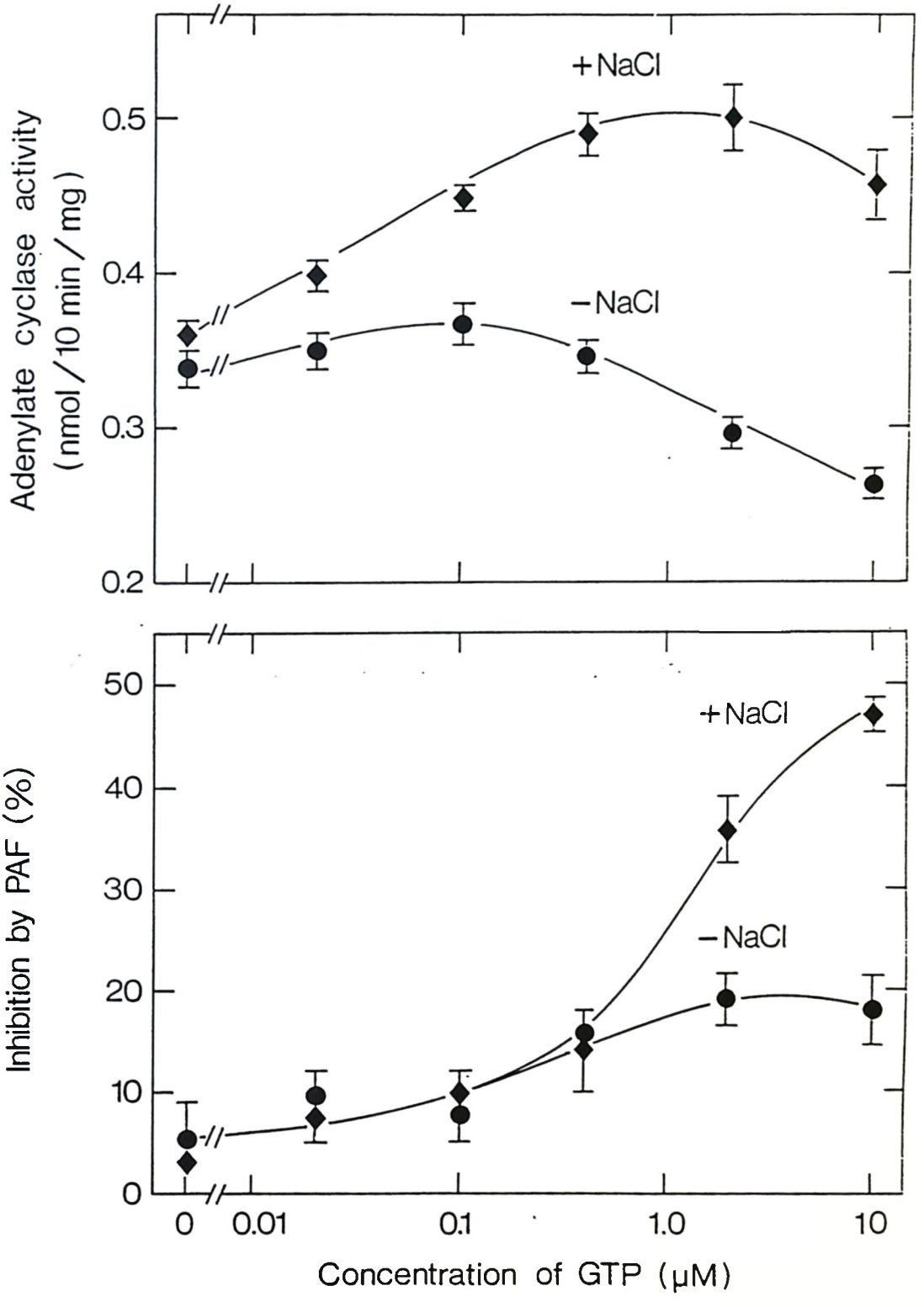
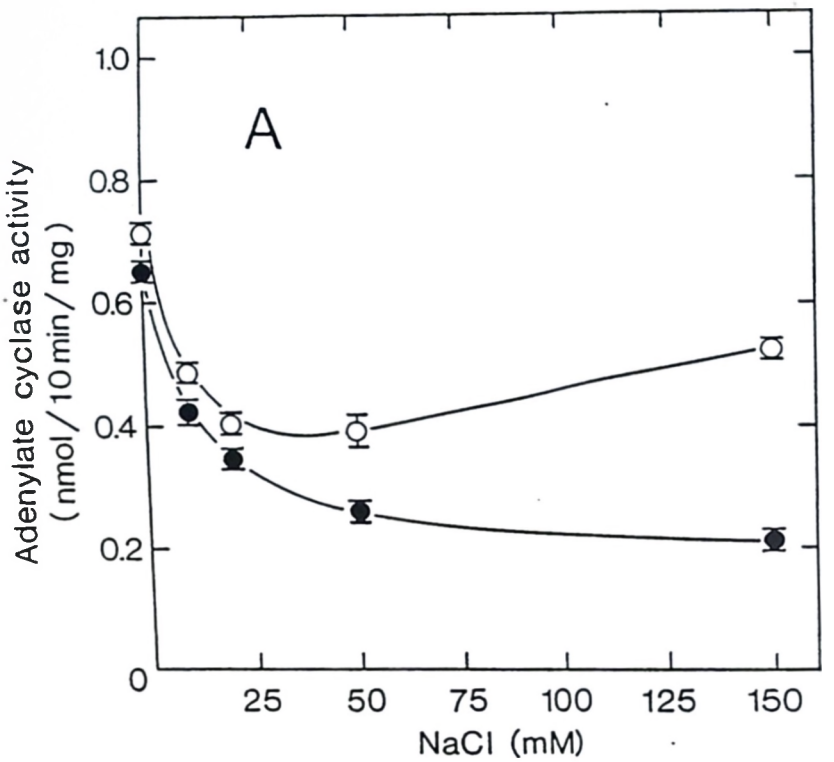
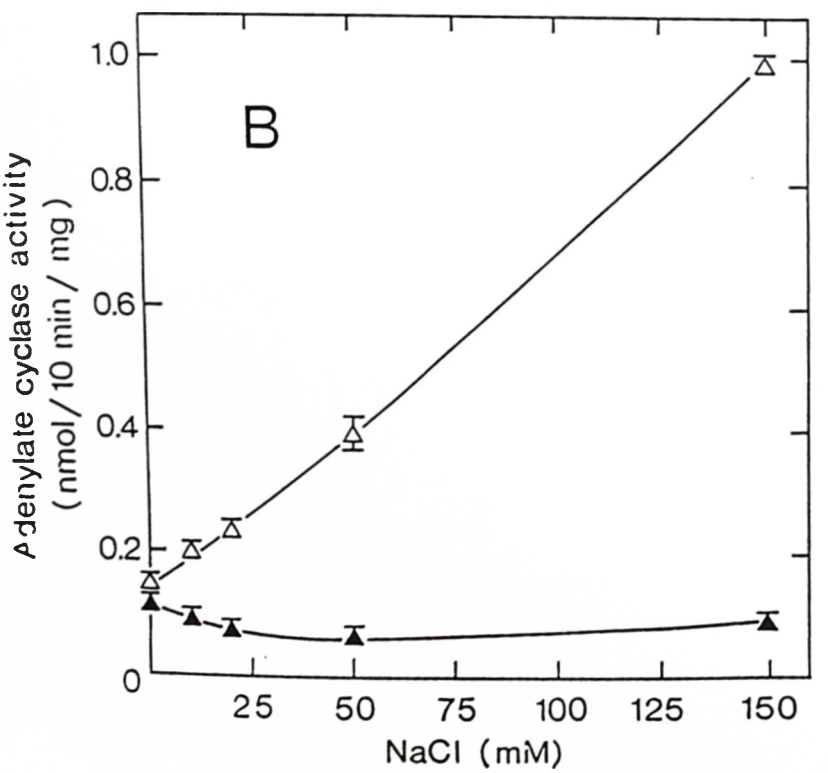
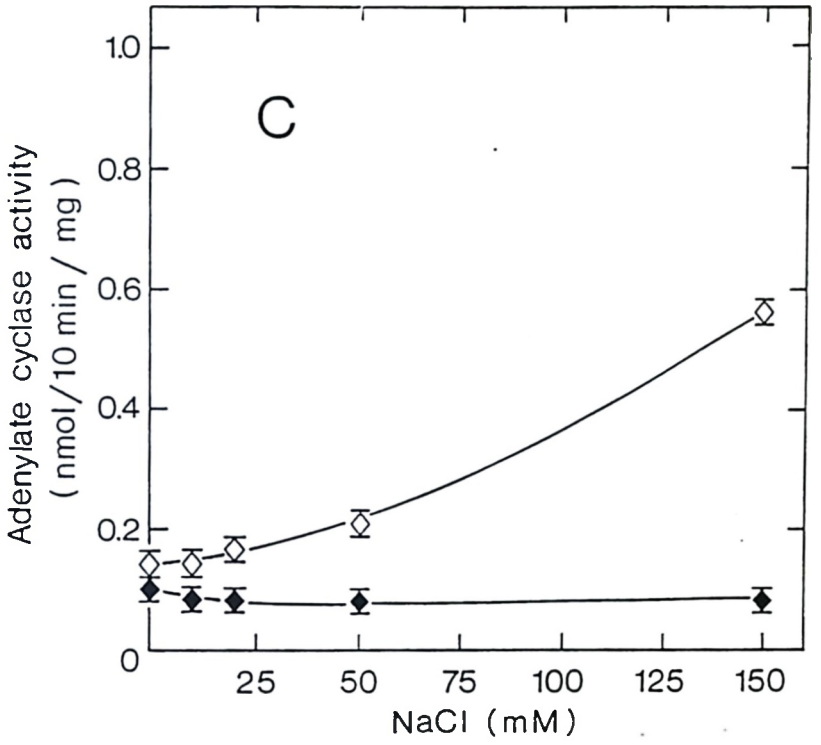

+NaCl
−NaCl
Adenylate cyclase activity
(nmol/10 min/mg)
0.5
0.4
0.3
0.2
+NaCl
−NaCl
Inhibition by PAF (%)
50
40
30
20
10
0
0
0.01
0.1
1.0
10
Concentration of GTP (μM)

Fig. 3.4. Effects of NaCl and PAF on the adenylate cyclase activities of particulate fractions prepared from rabbit platelets lysed under various conditions

Rabbit platelets lysed in 150 mM Tris-HCl (pH 7.4), containing (A) no other additions, (B) 5 mM EGTA or (C) 400 μM leupeptin were incubated for 15 min at 25°C before isolation of particulate fractions, as described in Section 2.2.7. Assays were carried out in the absence (open symbols) and presence (closed symbols) of 100 nM PAF. Inhibition of adenylate cyclase activity by this compound is shown in D, with additions during platelet lysis as follows: (●) none (from A); (▲) EGTA (from B); (◆) leupeptin (from C). Values for adenylate cyclase activity are means ± S.E.M. from triplicate assays (A, B and C) and (D) the effects of PAF are expressed as % inhibition ± S.E.Q.


A
Adenylate cyclase activity
(nmol/10 min/mg)
1.0
0.8
0.6
0.4
0.2
0
25
50
75
100
125
150
NaCl (mM)


B
Adenylate cyclase activity
(nmol/10 min/mg)
1.0
0.8
0.6
0.4
0.2
0
25
50
75
100
125
150
NaCl (mM)


C
Adenylate cyclase activity
(nmol/10 min / mg)
1.0
0.8
0.6
0.4
0.2
0
25
50
75
100
125
150
NaCl (mM)

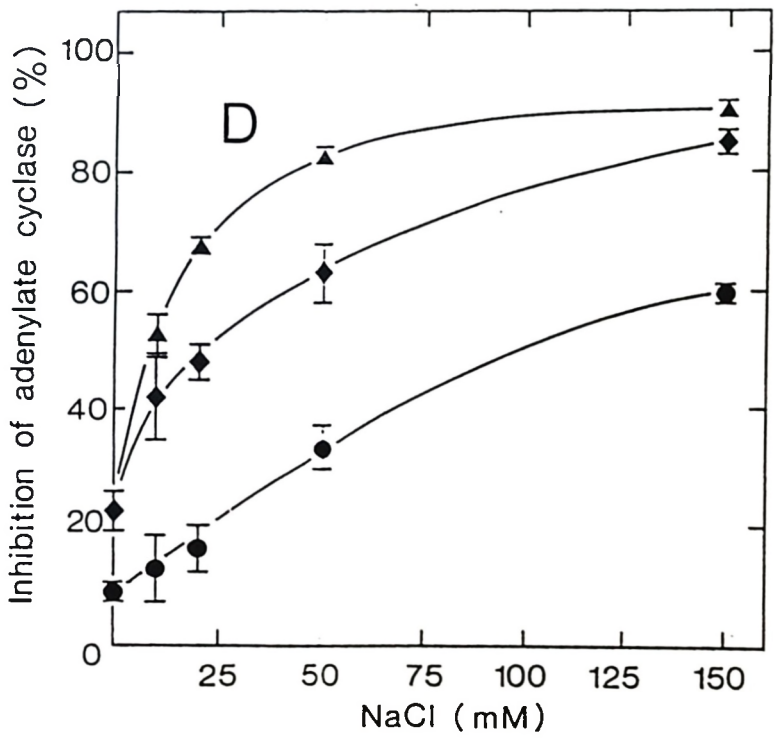
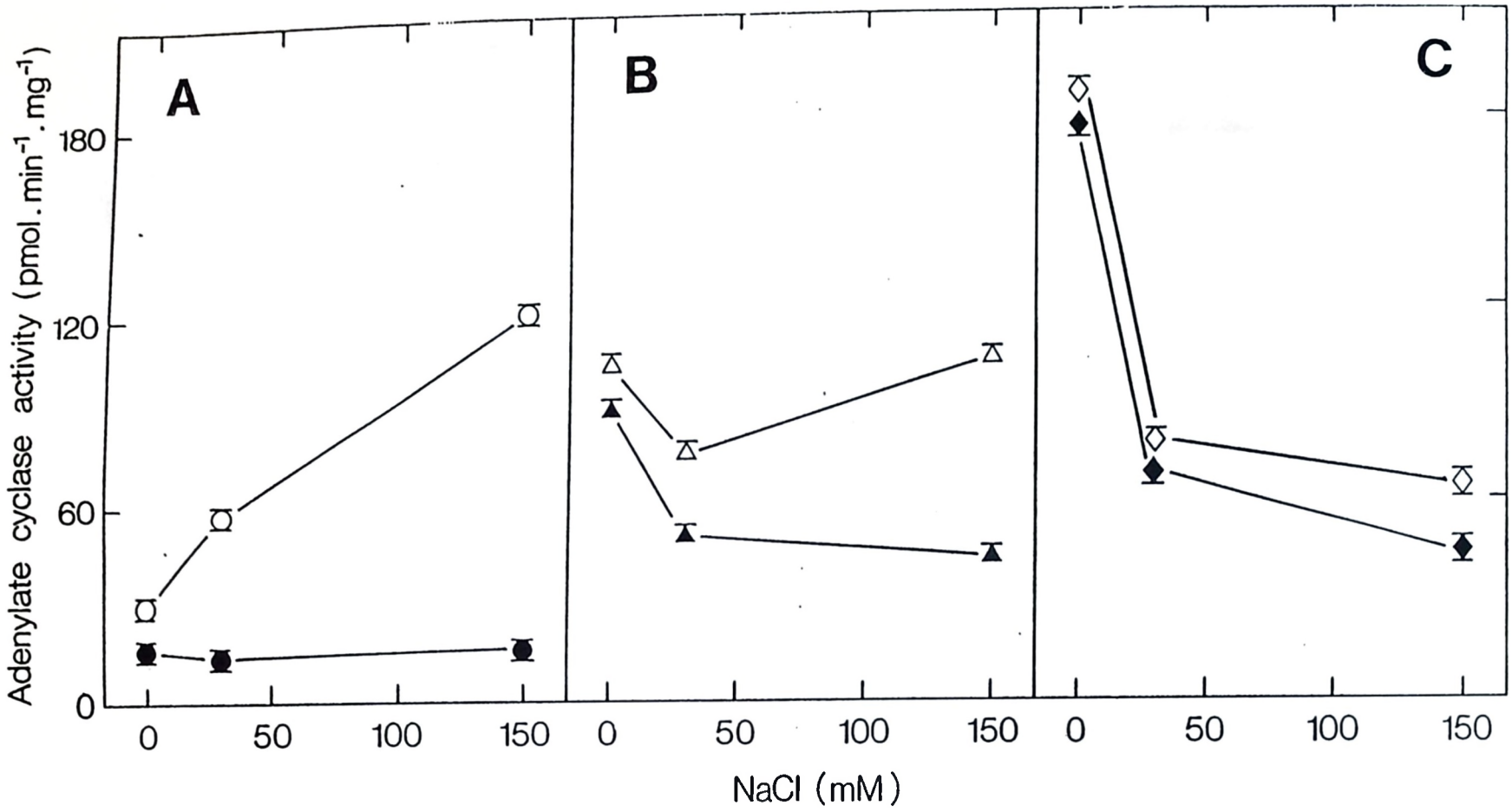

D
Inhibition of adenylate cyclase (%)
100
80
60
40
20
0
25
50
75
100
125
150
NaCl (mM)

Fig. 3.5. Effects of α-chymotrypsin on the actions of NaCl and PAF on the adenylate cyclase activities of rabbit platelet particulate fractions

Platelet lysate containing 5 mM EGTA, prepared as in Section 2.2.7., was incubated for 10 min at 25°C with no additions (A), 20 μg of α-chymotrypsin/ml (B) or 50 μg of α-chymotrypsin/ml (C). These incubations were terminated by addition of 500 μg of soybean trypsin inhibitor/ml and after 2 min the mixtures were cooled to 0°C. Particulate fractions were then isolated and washed twice by homogenization and centrifugation in buffer containing 10 mM Tris-HCl and 5 mM EGTA, pH 7.4. Assays were carried out in the absence (open symbols) and presence (closed symbols) of 100 nM PAF and values for adenylate cyclase activity are means ± S.E.M. from triplicate determinations. Inhibition of adenylate cyclase by PAF is shown in D for the following additions during incubations of the platelet lysate: (●) none (from A); (▲) 20 μg of α-chymotrypsin/ml (from B); (◆) 50 μg of α-chymotrypsin/ml (from C). Values are expressed as % inhibition ± S.E.Q.


A
B
C
Adenylate cyclase activity (pmol.min-1.mg-1)
180
120
60
0
0
50
100
150
NaCl (mM)

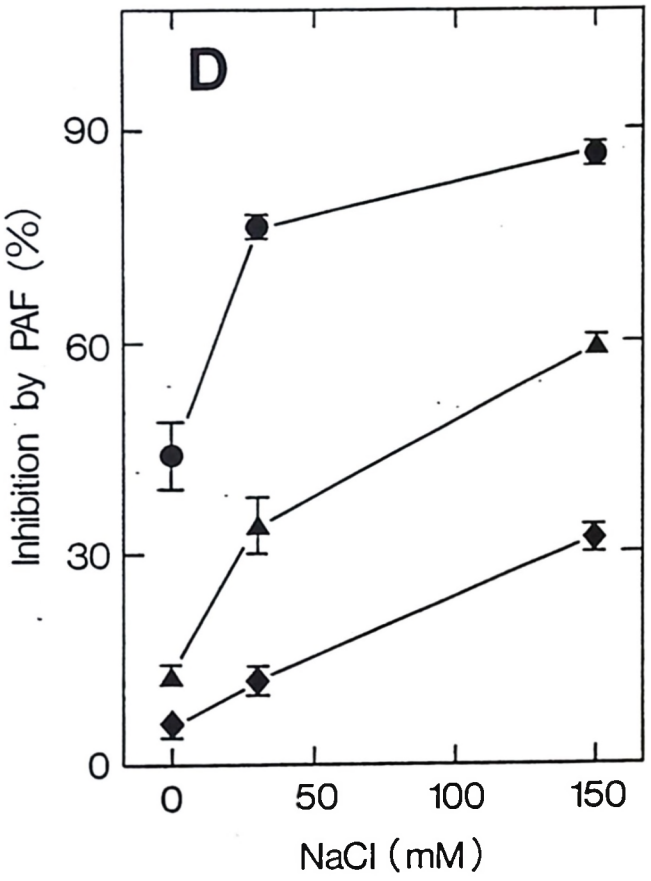

D
90
60
30
0
Inhibition by PAF (%)
0
50
100
150
NaCl (mM)

Table 3.1. Effects of NaCl on the inhibition of human platelet adenylate cyclase activity by PAF in incubation mixtures containing variable and constant total salt concentrations

Particulate fractions were prepared from human platelets lysed in the presence of 5 mM EGTA and were assayed for adenylate cyclase activity in the presence of increasing amounts of PAF at the indicated concentrations of NaCl. To demonstrate that the action of NaCl was not due to an increase in salt or Cl^- concentration, enzyme activity was also assayed in samples supplemented with choline chloride to bring the total salt concentration to 200 mM.

Additions		Adenylate cyclase activity at variable salt concentration		Adenylate cyclase activity at constant salt concentration	
NaCl (mM)	PAF (nM)	nmol/10 min per mg protein	% of control	nmol/10 min per mg protein	% of control
None	None	0.382 ± 0.024	–	0.393 ± 0.025	–
	0.01	0.371 ± 0.020	103 ± 8	0.395 ± 0.017	100 ± 8
	0.10	0.333 ± 0.022	87 ± 8	0.358 ± 0.032	91 ± 10
	1.0	0.244 ± 0.005	64 ± 4	0.256 ± 0.007	65 ± 4
	10	0.253 ± 0.015	66 ± 6	0.232 ± 0.007	59 ± 4
	100	0.222 ± 0.010	58 ± 4	0.218 ± 0.010	56 ± 4
40 mM	None	0.689 ± 0.040	–	0.711 ± 0.046	–
	0.01	0.679 ± 0.033	99 ± 7	0.723 ± 0.056	102 ± 10
	0.10	0.645 ± 0.042	94 ± 8	0.672 ± 0.030	94 ± 7
	1.0	0.523 ± 0.023	76 ± 5	0.523 ± 0.026	74 ± 6
	10	0.292 ± 0.015	42 ± 3	0.301 ± 0.009	42 ± 3
	100	0.240 ± 0.022	35 ± 4	0.237 ± 0.011	33 ± 3
100 mM	None	0.896 ± 0.047	–	1.054 ± 0.132	–
	0.01	0.895 ± 0.055	100 ± 8	0.878 ± 0.041	84 ± 11
	0.10	0.849 ± 0.046	95 ± 7	0.877 ± 0.055	83 ± 12
	1.0	0.768 ± 0.044	86 ± 7	0.829 ± 0.052	79 ± 11
	10	0.443 ± 0.024	49 ± 4	0.474 ± 0.020	45 ± 6
	100	0.288 ± 0.009	32 ± 2	0.300 ± 0.010	29 ± 4
200 mM	None	1.019 ± 0.073	–		
	0.01	1.004 ± 0.049	99 ± 9		
	0.10	1.030 ± 0.060	101 ± 9		
	1.0	0.973 ± 0.055	96 ± 9		
	10	0.790 ± 0.47	78 ± 7		
	100	0.477 ± 0.02	47 ± 4		

Table 3.2. Role of GTP in the actions of NaCl and PAF on adenylate cyclase activity in non-proteolysed and proteolysed rabbit platelet particulate fractions

Platelet lysate containing 5 mM EGTA, prepared as described in Section 2.2.7., was incubated for 15 min at 25°C either with no addition or with sufficient $CaCl_2$ to give a final $[Ca^{2+}]$ of 200 μM. Washed platelet particulate fractions were then prepared by centrifugation and resuspension in hypotonic buffer. Adenylate cyclase assays were carried out in the absence and presence of 100 nM PAF at the indicated concentrations of NaCl and GTP. Values for adenylate cyclase activity are means ± S.E.M. from triplicate determinations. To demonstrate the dependence on GTP of the action of NaCl on adenylate cyclase activity, the difference between the enzyme activities in the absence and presence of 10 μM GTP was expressed as a % of the activity in the absence of GTP (mean change ± S.E.Q. given). Values for the % inhibition by PAF are also means ± S.E.Q.

3.2

Enzyme preparation	NaCl (mM)	GTP (μM)	Adenylate cyclase activity in the absence of PAF		Adenylate cyclase activity in the presence of PAF	
			nmol/10 min per mg protein	Change caused by GTP (%)	nmol/10 min per mg protein	Inhibition by PAF (%)
No $CaCl_2$	0	0	0.590 ± 0.014	–	0.490 ± 0.021	17 ± 4
		10	0.366 ± 0.011	-38 ± 3	0.290 ± 0.033	21 ± 3
	30	0	0.736 ± 0.018	–	0.483 ± 0.016	34 ± 3
		10	0.630 ± 0.008	-14 ± 3	0.187 ± 0.006	70 ± 1
	150	0	1.222 ± 0.002	–	0.746 ± 0.024	39 ± 2
		10	1.927 ± 0.035	+58 ± 3	0.243 ± 0.010	87 ± 1
$CaCl_2$	0	0	0.955 ± 0.018	–	0.806 ± 0.016	16 ± 2
		10	0.803 ± 0.035	-16 ± 4	0.743 ± 0.010	7 ± 4
	30	0	0.807 ± 0.015	–	0.548 ± 0.001	32 ± 1
		10	0.714 ± 0.002	-12 ± 2	0.362 ± 0.005	49 ± 1
	150	0	1.167 ± 0.015	–	0.743 ± 0.023	36 ± 2
		10	1.301 ± 0.011	+12 ± 2	0.315 ± 0.005	76 ± 1

Table 3.3. Effects of different monovalent cations on the adenylate cyclase activities of non-proteolysed and proteolysed rabbit platelet particulate fractions; modification of the inhibitory action of PAF

Rabbit platelets were lysed in 150 mM Tris-HCl (pH 7.4) in the presence and absence of 5 mM EGTA and then incubated for 15 min at 25°C before isolation of particulate fractions. Adenylate cyclase assays were carried out in the absence and presence of 100 nM PAF at the indicated concentrations of salts. To show the effect of each monovalent cation in the absence of PAF, the difference in enzyme activity between samples incubated with and without the specified salt was expressed as a percentage of the activity in the absence of the salt (mean change ± S.E.Q. given). Values for the % inhibition by PAF at the indicated salt concentrations are also means ± S.E.Q.

3.3.

Enzyme preparation	Salt	Concn. (mM)	Adenylate cyclase activity in the absence of PAF		Adenylate cyclase activity in the presence of PAF	
			nmol/10 min per mg protein	change (%)	nmol/10 min per mg protein	Inhibition by PAF (%)
+ EGTA	None	0	0.196 ± 0.005	–	0.146 ± 0.001	26 ± 2
	NaCl	30	0.405 ± 0.002	+107 ± 5	0.105 ± 0.003	74 ± 1
		150	1.155 ± 0.027	+489 ± 20	0.121 ± 0.003	90 ± 0
	LiCl	30	0.240 ± 0.006	+ 22 ± 4	0.135 ± 0.005	44 ± 3
		150	0.358 ± 0.005	+ 83 ± 5	0.131 ± 0.004	63 ± 1
	KCl	30	0.184 ± 0.002	- 6 ± 3	0.123 ± 0.001	33 ± 1
		150	0.170 ± 0.001	- 13 ± 2	0.107 ± 0.000	37 ± 0
	Choline-Cl	30	0.197 ± 0.002	+ 1 ± 3	0.134 ± 0.006	32 ± 2
		150	0.207 ± 0.004	0 ± 3	0.138 ± 0.003	33 ± 2
- EGTA	None	0	0.664 ± 0.003	–	0.597 ± 0.002	10 ± 1
	NaCl	30	0.419 ± 0.008	- 37 ± 1	0.305 ± 0.005	27 ± 2
		150	0.782 ± 0.018	+ 18 ± 3	0.236 ± 0.002	70 ± 1
	LiCl	30	0.551 ± 0.011	- 17 ± 2	0.500 ± 0.008	9 ± 2
		150	0.453 ± 0.018	- 14 ± 3	0.360 ± 0.006	21 ± 3
	KCl	30	0.571 ± 0.009	- 14 ± 1	0.526 ± 0.003	8 ± 2
		150	0.421 ± 0.003	- 37 ± 1	0.387 ± 0.014	8 ± 3
	Choline-Cl	30	0.633 ± 0.010	- 5 ± 2	0.595 ± 0.006	6 ± 2
		150	0.609 ± 0.019	- 8 ± 3	0.574 ± 0.020	6 ± 4

Chapter 4

Simultaneous measurement of agonist-induced changes in the adenylate cyclase and phospholipase C activities of isolated rabbit platelet membranes

4.1. *Introduction*

4.1.1. *Characterization of the G protein mediating activation of phospholipase C by platelet agonists*

In many cells including platelets, some agonists act both to inhibit adenylate cyclase and activate phospholipase C in a GTP-dependent manner (Lapetina, 1983; Aktories and Jakobs, 1984; MacIntyre *et al.*, 1985b; Heller Brown and Brown, 1984; Garcia-Sainz, 1987; Trimble *et al.*, 1987). In the platelet these include thrombin and PAF. The ability of these agonists to affect the activities of more than one target enzyme raises the possibility that distinct receptors and/or G proteins couple to the different effector systems. To study this question, it is necessary to demonstrate differential effects on adenylate cyclase and phospholipase C of agents that affect the activities of specific components of the signal transduction pathway.

Treatment of human platelet membranes with pertussis toxin attenuated the inhibition of adenylate cyclase activity by epinephrine (Aktories *et al.*, 1983b) or thrombin (Aktories and Jakobs, 1984), suggesting that receptors for these agonists were coupled to adenylate cyclase by G_i. However, pertussis toxin only partially reduced the ability of PAF, U44069 (Houslay *et al.*,

1986b) and thrombin (Grandt *et al.*, 1986; Houslay *et al.*, 1986a) to stimulate human platelet membrane GTPase activity. Since epinephrine does not activate phospholipase C (Siess *et al.*, 1984) and pertussis toxin abolished epinephrine-stimulated GTPase activity under the same conditions, these investigators postulated that G_i was not the G-protein responsible for mediating GTP-dependent hormonal stimulation of phospholipase C in human platelets. Studies of the effects of pertussis toxin on intact platelets are not possible probably because they do not possess receptors for the binding component of the toxin, which is required for internalization of the S_1 subunit containing the ADP-ribosyltransferase activity (Ui, 1984). Therefore, various workers have used human platelets permeabilized with saponin to allow penetration of the toxin. These studies have shown both increases (Lapetina, 1986) and decreases (Brass *et al.*, 1986) in thrombin-stimulated phospholipase C activity in suspensions of pertussis toxin-treated permeabilized platelets. In a later study, Brass *et al.* (1987) found that although the activation of phospholipase C by U46619 or thrombin was blocked by GDPβS, the action of U46619 was unaffected by pertussis toxin. Additional evidence in support of a role for a pertussis toxin substrate in activation of phospholipase C by thrombin was obtained by combining platelet plasma membranes with dense tubular membranes containing Ca^{2+} (O'Rourke *et al.*, 1987). In this study, pertussis toxin blocked the release of Ca^{2+} induced by thrombin and GTP. However,

the involvement of a specific G_i in activation of phospholipase C by thrombin was obscured by the observation of two pertussis toxin substrates in platelets (O'Rourke *et al.*, 1987). It now appears that in many cells the α_i-subunit of G_i is not the only substrate for pertussis toxin (Neer *et al.*, 1984; Gierschik *et al.*, 1986; Rapiejko *et al.*, 1986; Katada *et al.*, 1987a), including platelets (O'Rourke *et al.*, 1987). Immunochemical and cDNA sequencing studies of GTP-binding proteins have established that at least three G_i-related pertussis toxin substrates exist (Jones and Reed, 1987; Itoh *et al.*, 1988).

4.1.2. *Objectives of this study*

The purpose of this study was to determine whether the G protein mediating hormonal inhibition of adenylate cyclase (G_i) couples receptors for PAF and thrombin to phospholipase C in rabbit platelet membranes. An assay that allows measurement of adenylate cyclase and phospholipase C activities under identical conditions in particulate fractions from the same population of rabbit platelets was developed in order to determine the effect of inactivation of α_i by pertussis toxin or NEM on the abilities of the platelet agonists, PAF, thrombin and epinephrine to interact with these two effector systems.

4.2. *Results*

4.2.1. *Simultaneous assay of adenylate cyclase and phospholipase C activities in rabbit platelet particulate fraction*

By modification of previous methods, it proved possible to measure the formation of [^{32}P]cAMP from [α-^{32}P]ATP and the release of [^{3}H]inositol phosphates from endogenous [^{3}H]phosphoinositides under essentially identical experimental conditions. Before the development of this combination assay, the rabbit platelet particulate fractions used in this laboratory for assay of adenylate cyclase and phospholipase C were prepared according to Section 2.2.7. and Hrbolich *et al.* (1987), respectively. Thus, for measurement of adenylate cyclase activity alone, platelet particulate fractions were washed by resuspension and homogenization in a solution containing 10 mM Tris-HCl (pH 7.4) and 5 mM EGTA. However, preservation of polyphosphoinositides in platelet membranes used for measurement of phospholipase C required addition of ATP and $MgCl_2$ to the washing solution (Hrbolich *et al.*, 1987). The assay conditions finally adopted for the combination system also mainly reflected those optimal for phospholipase C, activity but with some modifications necessary to accommodate the measurement of adenylate cyclase (see Table 4.1.). Thus, the incubation medium differed from that used for measurement of phospholipase C alone by replacement of KCl with NaCl (100 mM) and a

decrease in the ATP concentration from 1.23 mM to 0.46 mM. These changes were made because the inhibition of adenylate cyclase by agonists was potentiated by NaCl (Chapter 3) and because the higher ATP concentration would waste [α-^{32}P]ATP. In addition, a number of compounds required for optimal activity of adenylate cyclase were added to the incubation medium used in the combination assay. These included 4 mM CP and 20 units CPK/ml (to preserve substrate), 0.4 mM DTT and 1 mg of BSA/ml. IBMX was also included to block the breakdown of the cAMP formed (see Table 4.1.). Changes to the original adenylate cyclase assay medium included: a decrease in the assay temperature from 30°C to 25°C, a decrease in the $MgCl_2$ concentration from 5 mM to 0.96 mM, replacement of [^{3}H]ATP by [α-^{32}P]ATP, omission of unlabelled cAMP and replacement of 75 mM Tris-HCl by 25 mM Hepes, pH 7.4. Cyclic AMP was ommitted because it has been shown to inhibit the activation of platelet phospholipase C (Nishizuka, 1984) and the $MgCl_2$ concentration was decreased to that of the phospholipase C assay (Hrbolich *et al.*, 1987) to reduce the breakdown of inositol phosphates (Downes and Michell, 1981, see also Table 4.2.). [^{3}H]Inositol phosphates and [^{32}P]cAMP could not be isolated from the same samples because [^{32}P]adenine nucleotides co-eluted with [^{3}H]inositol phosphates. The only difference in the assays for adenylate cyclase and phospholipase C activities in the combination system was therefore the inclusion of 2 μCi of [α-^{32}P]ATP in the samples from which [^{32}P]cAMP was isolated.

The above combination assay demonstrated that addition of PAF and GTP to rabbit platelet membranes caused a simultaneous inhibition of adenylate cyclase and activation of phospholipase C (Table 4.2). The effects of unlabelled cAMP and of two $MgCl_2$ concentrations on the assay were investigated in a preliminary experiment (Table 4.2.). In the presence of 4.8 mM $MgCl_2$, [^{3}H]IP_2 formation was decreased and IP_1 formation increased relative to the amounts accumulating with 0.96 mM $MgCl_2$, suggesting that inositol phosphate degradation was enhanced by the higher concentration of $MgCl_2$, as expected (Downes and Michell, 1981). Very little [^{3}H]IP_3 accumulated under either condition, as previously found by Hrbolich *et al.* (1987). Thus, at the lower $MgCl_2$ concentration, measurement of [^{3}H]IP_2 alone provided a valid assay of phospholipase C activity. In contrast, [^{32}P]cAMP formation was optimal at the higher concentration of $MgCl_2$. However, since the lower $MgCl_2$ concentration (Mg^{2+}_{free}, 0.52 mM) is closer to the physiological range (Bockaert *et al.*, 1984; Maguire, 1984) and the responsiveness of adenylate cyclase to PAF and GTP was not reduced in percentage terms at this concentration of Mg^{2+}_{free}, 0.96 mM $MgCl_2$ was used in the combination system. Addition of 1 mM cAMP slightly decreased [^{3}H]IP_2 formation and this inhibitory effect was the greatest in the presence of 4.8 mM $MgCl_2$. Since no decrease in the amounts of [^{32}P]cAMP accumulating or in the percent inhibition of adenylate cyclase by PAF and GTP were detected in the absence of exogenous cAMP, this compound was omitted from the

combination assay. The results (Table 4.2) thus showed that it was possible to measure both phospholipase C and adenylate cyclase in a single preparation of [^{3}H]inositol-labelled rabbit platelet membranes.

4.2.2. *Effects of PAF, thrombin and epinephrine on adenylate cyclase and phospholipase C activities*

Utilizing the above assay conditions, the abilities of different agonists to inhibit adenylate cyclase and activate phospholipase C were compared (Table 4.3.). Both PAF and thrombin were potent inhibitors of adenylate cyclase and activators of phospholipase C. In the presence of 100 mM NaCl and 10 μM GTP, 1 μM PAF inhibited rabbit platelet adenylate cyclase by 92 $\pm$ 1% (mean value $\pm$ S.E.M., 7 expts.) and increased [^{3}H]IP_2 formation by 526 $\pm$ 174% (mean value $\pm$ S.E.M., 7 expts.). The increases in [^{3}H]IP_2 accumulation caused by PAF ranged from 120% to 1163% in different experiments, though within experiments little difference was seen between replicate incubations. The source of this variation is not known, but it was not specific to PAF and was unrelated to the uptake of [^{3}H]inositol by the rabbit platelets. In two experiments with samples containing 100 mM NaCl and 10 μM GTP, 2 units of thrombin/ml inhibited adenylate cyclase by 84 $\pm$ 1% and 86 $\pm$ 1% and stimulated [^{3}H]IP_2 formation by 614 $\pm$ 37% and 216 $\pm$ 22% (mean values $\pm$ S.E.Q.).

In contrast to the dual action of PAF and thrombin on rabbit platelet particulate fractions, 5 μM epinephrine had no effect on phospholipase C activity in the same preparations. In the presence of 100 mM NaCl and 10 μM GTP, 5 μM epinephrine (mixed with 10 μM propranolol) inhibited adenylate cyclase activity by 75 $\pm$ 2% (mean value $\pm$ S.E.M., 6 expts.) but increased [^{3}H]IP_2 formation by only 12 $\pm$ 8% (mean value $\pm$ S.E.M., 6 expts.). The amounts of [^{3}H]IP_2 formed in the presence of epinephrine and GTP were not significantly different from the amounts formed in control samples containing GTP alone ($P > 0.05$; paired t test). This did not simply reflect a weak agonistic effect of epinephrine, since addition of 0.2 units of thrombin/ml stimulated [^{3}H]IP_2 formation, though it decreased [^{32}P]cAMP formation to a lesser extent than did epinephrine (Table 4.3.). In contrast to the failure of epinephrine alone to activate phospholipase C in rabbit platelet membranes, this agonist enhanced [^{3}H]IP_2 formation caused by suboptimal doses of either PAF or thrombin (Table 4.3), suggesting that under certain conditions epinephrine may potentiate the activation of this enzyme. Thus, in this experiment, epinephrine significantly increased the formation of [^{3}H]IP_2 by 20 nM PAF ($P < 0.05$, unpaired t test) and had a marginally significant effect on the action of 0.2 units of thrombin/ml ($P < 0.10$, unpaired t test).

4.2.3. *GTP dependence of the actions of PAF on adenylate cyclase and phospholipase C*

When rabbit platelet particulate fractions that had been washed twice with hypotonic buffer were incubated with 10 μM GTP (in the presence of 100 mM NaCl), there was no significant change in adenylate cyclase activity ($P > 0.05$, paired *t* test, 7 expts.). In contrast, [^{3}H]IP_2 formation measured simultaneously was stimulated 35 $\pm$ 7% by 10 μM GTP (mean value $\pm$ S.E.M., 7 expts.), which is comparable to the value obtained by Hrbolich *et al.* (1987). GTP enhanced both the inhibition of adenylate cyclase and stimulation of phospholipase C caused by PAF (see Fig. 4.1.). The inhibition of adenylate cyclase by 2 nM PAF increased from 31 $\pm$ 6% to 40 $\pm$ 7% and that caused by 1 μM PAF from 82 $\pm$ 2% to 90 $\pm$ 3% on addition of 10 μM GTP (mean values $\pm$ S.E.M., 5 expts.). Although the ability of GTP to enhance the inhibition of adenylate cyclase by 1 μM PAF was significant ($P < 0.05$, paired *t* test, 5 expts.), PAF was clearly a potent inhibitor of adenylate cyclase even in the absence of added GTP in this system. The effect of GTP on the inhibition of adenylate cyclase by 2 nM PAF was only marginally significant ($P < 0.10$ and > 0.05, paired *t* test, 5 expts.). These results contrast with the greater dependence of the inhibitory effect of PAF on GTP in previous studies in which adenylate cyclase alone was measured (Table 3.2). Thus, in the presence of 150 mM NaCl, 100 nM PAF caused a 40% inhibition of adenylate cyclase that

was increased to 90% upon addition of 10 μM GTP (see Table 3.2). This discrepancy may be a consequence of less efficient removal of GTP from the platelet particulate fractions or the use of a lower Mg^{2+}_{free} concentration in the combination assay.

The stimulatory action of PAF on phospholipase C activity was much more highly dependent on added GTP (Fig. 4.1). The increase in phospholipase C activity caused by 2 nM PAF was enhanced from 17 $\pm$ 10% to 57 $\pm$ 26% and that caused by 1 μM PAF from 136 $\pm$ 71% to 478 $\pm$ 186% upon addition of 10 μM GTP (mean values $\pm$ S.E.M., 5 expts.). The Wilcoxon signed rank test showed that the ability of GTP to increase the stimulation of phospholipase C by both 2 nM and 1 μM PAF was statistically significant ($P < 0.02$, 5 expts.). These results suggest that there is a difference in the dependencies of the actions of PAF on adenylate cyclase and phospholipase C on exogenous GTP (see Fig. 4.1). Presumably, contaminating GTP was almost sufficient for maximal inhibition of adenylate cyclase but was insufficient to support marked activation of phospholipase C. This could reflect roles for two G proteins with different affinities for GTP in the interactions of PAF receptor(s) with these two effector systems.

The dose-response curves for PAF in the combination assay (Fig. 4.1.) demonstrated that an approx. 5-fold higher concentration of PAF (~10 nM) was required for half-maximal activation of

phospholipase C than for half-maximal inhibition of adenylate cyclase (~2 nM). It is possible that more PAF receptors must be occupied for optimal activation of phospholipase C than for maximal inhibition of adenylate cyclase. Alternatively, distinct receptors with slightly different affinities for PAF could be coupled to adenylate cyclase and phospholipase C. This finding is also supported by less complete data in Tables 4.3. and 4.6.

4.2.4. *[^{32}P]ADP-ribosylation of platelet proteins by pertussis toxin*

In rabbit platelet membranes treated with pertussis toxin and [^{32}P]NAD^+, three ^{32}P-labelled proteins in the 38-42 kDa range were observed with the highest molecular mass protein being the most heavily labelled (Fig. 4.2). Incubation of platelet particulate fractions with and without pertussis toxin in the presence of 1 mM NAD^+ followed by a second exposure to the toxin in a medium containing 10 μM [^{32}P]NAD^+, allowed quantitation of the total extent of ADP-ribosylation of the pertussis toxin substrates (Olate *et al.*, 1984). Inclusion of 5 mM EGTA, 200 μg leupeptin/ml, 1 mM phenylmethylsulfonyl fluoride, 1 mM benzamidine and 100 μg of soybean trypsin inhibitor/ml in the media used for isolation, washing and treatment of rabbit platelet particulate fractions with pertussis toxin did not alter the pattern of [^{32}P]ADP-ribosylation of platelet proteins (Fig. 4.3). However, since the amount of ^{32}P

incorporated into the platelet proteins was less in the membranes exposed to pertussis toxin in the presence of the proteolytic inhibitors, it appears that one or more of these compounds inhibited the ADP-ribosyltransferase activity of the toxin. These findings suggest that the lower molecular mass ^{32}P-labelled proteins in rabbit platelet membranes were not proteolytic fragments of G_i.

4.2.5. *Effects of treatment of rabbit platelet membranes with pertussis toxin on agonist-induced inhibition of adenylate cyclase and stimulation of phospholipase C*

Studies with the holotoxin. When rabbit platelet particulate fractions were incubated with 40-50 μg of activated pertussis toxin/ml for 30 min at 37°C, the 38-42 kDa toxin substrates were ADP-ribosylated to between 30 and 84% (Table 4.4.). The source of this variation remains unclear. Differences in the ADP-ribosyltransferase activities of the batches of toxin used, in the activation process or in the susceptibility of the rabbit platelet membranes to the toxin could account for the discrepant results. Measurement of the adenylate cyclase and phospholipase C activities of pertussis toxin-treated rabbit platelet particulate fractions using the combination assay, showed that pertussis toxin had marked inhibitory effects on the basal activities of these enzymes (Table 4.4.). In the three experiments in Table 4.4., formation of [^{32}P]cAMP and [^{3}H]IP_2 was decreased by pertussis

toxin by 41-54% and 14-82%, respectively.

A consistent effect of treatment of rabbit platelet membranes with pertussis toxin was a decrease in the percent inhibition of adenylate cyclase by PAF and GTP or, particularly, epinephrine and GTP. This was attributable to increased enzyme activity in the presence of PAF or epinephrine, as well as the decrease in control adenylate cyclase activity (Table 4.4). Simultaneous measurement of phospholipase C activities, showed that ADP-ribosylation of 30 - 84% of the pertussis toxin substrates had no effect on the percent stimulation of $[^3H]IP_2$ formation caused by PAF. However, there were decreases in the absolute amounts of $[^3H]IP_2$ formed in the presence of PAF and GTP that were roughly proportional to the decreases in basal $[^3H]IP_2$ formation.

Studies with the S_1 subunit of pertussis toxin.

Purified S_1 subunit was used in an attempt to obtain more consistent and complete ADP-ribosylation of platelet pertussis toxin substrates. Use of S_1 subunits should also avoid the possibility of confounding effects of the other toxin subunits (Banga *et al.*, 1987). Treatment of rabbit platelet particulate fraction with 10 μg of S_1 subunit/ml for 30 min at 37°C, ADP-ribosylated 91-94% of the platelet 38-42 kDa pertussis toxin substrates (Table 4.5). Under these conditions $[^{32}P]$cAMP and $[^3H]IP_2$ formation were decreased by 27-37% and 19-29%, respectively (Table 4.5.).

These inhibitions of basal enzyme activities were smaller and less variable than observed with the holotoxin. When the experiments with holotoxin and S_1 subunit are both considered, the inhibitions of basal adenylate cyclase and phospholipase C activities did not correlate with the degree of ADP-ribosylation of platelet proteins. However, assuming the S_1 subunit has a single effect, ADP-ribosylation of platelet polypeptides does appear to contribute to this inhibitory process.

Rabbit platelet particulate fractions with 91-94% of their 38-42 kDa pertussis toxin substrates ADP-ribosylated by the S_1 subunit of pertussis toxin showed significant but variable decreases in the inhibition of adenylate cyclase activity by PAF and GTP or by epinephrine and GTP. However, in no experiment were the inhibitory actions of these agents blocked completely, despite the nearly complete ADP-ribosylation of the pertussis toxin substrates. In aggregate, the decreases in the inhibitory effect of epinephrine caused by pertussis holotoxin and purified S_1 subunit were greater than those observed with PAF ($P < 0.10$ and > 0.05; paired t test, 5 expts.) suggesting that PAF at least may inhibit adenylate cyclase through pertussis toxin-sensitive and insensitive pathways. Measurement of phospholipase C under identical conditions showed no inhibitory effect of the S_1 subunit on the percent stimulation of $[^3H]IP_2$ formation caused by PAF. As with the holotoxin, the decreases observed in the absolute amounts of $[^3H]IP_2$ formed

from rabbit platelet membranes treated with the S_1 subunit paralleled the inhibitory effect on basal enzyme activity.

Conclusions. The above studies with rabbit platelet membranes treated with either activated pertussis holotoxin or purified S_1 subunits suggest either that the G protein mediating activation of phospholipase C by PAF is not a pertussis toxin subtrate or that a very small fraction of the pertussis toxin substrates available (<10%) is sufficient to mediate activation of the enzyme. In addition, they indicate that in the rabbit platelet, inhibition of adenylate cyclase by PAF may be mediated by a pertussis toxin-insensitive G protein, as well as by one or more of the pertussis toxin substrates.

4.2.6. *Effects of treatment of rabbit platelet membranes with NEM on the actions of GTP, PAF and epinephrine on adenylate cyclase and phospholipase C*

When the same preparation of rabbit platelet membranes was assayed for both adenylate cyclase and phospholipase C, NaCl and KCl had different effects on the activities of these two enzymes. Addition of 100 mM NaCl increased [^{32}P]cAMP formation 106 ± 32% (mean value ± S.E.M., 3 expts.) above that observed in rabbit platelet membrane suspensions containing 100 mM KCl. In contrast, 100 mM KCl increased [^{3}H]IP_2 formation by 89 ± 21% (mean value ±

S.E.M., 3 expts.) above that observed in membrane suspensions containg 100 mM NaCl. Addition of 10 μM GTP to rabbit platelet membranes stimulated [^{3}H]IP_2 formation in suspensions containing either NaCl or KCl. Investigation of the effect of PAF on adenylate cyclase in the combination assay, using membrane suspensions containing NaCl or KCl, showed that the compound was equally effective in the presence of either salt (Table 4.6.). Thus, in this study, PAF inhibited adenylate cyclase in the platelet membrane suspensions containing KCl more potently than observed with the earlier assay (compare Tables 4.6. and 3.3.). This could reflect the different Mg^{2+} concentrations used, as low Mg^{2+} promotes inhibition of adenylate cyclase (Smith and Harden, 1985; Bockaert *et al.*, 1984). High PAF concentrations (1 μM) caused a greater percentage stimulation of [^{3}H]IP_2 formation in suspensions containing NaCl than KCl. This was largely attributable to the higher basal phospholipase C activity in the presence of the latter salt. On the other hand, NaCl appeared to diminish the ability of low PAF concentrations to stimulate phospholipase C (Table 4.6.), as observed with adenylate cyclase (Table 4.6. and Chapter 3).

To examine further the possibility that a G_i-related protein was involved in the activation of phospholipase C by PAF, rabbit platelet membrane suspensions were treated with NEM to alkylate the cysteine residue that is ADP-ribosylated by pertussis toxin (Ui and Katada, 1987). Preincubation of rabbit platelet

membranes with 50 μM NEM for 15 min at 25°C was found to decrease the basal adenylate cyclase activities of membranes assayed in the presence of either NaCl or KCl, though a more pronounced inhibitory action of NEM was observed in the presence of NaCl. Whereas NEM also decreased adenylate cyclase activity measured in the presence of GTP when NaCl was present, it increased this activity when KCl was present (Table 4.6.). Moreover, although 10 μM GTP had little effect on or inhibited adenylate cyclase in control membranes (depending on whether NaCl or KCl was present), it stimulated the enzyme in NEM-treated membranes. These results are most easily explained by inhibition of the activity of G_i by NEM treatment (Jakobs *et al.*, 1982). NEM also inhibited basal phospholipase C activity but to almost the same extent in the presence of NaCl or KCl. Moreover, the difference between the effect of GTP on adenylate cyclase in control and NEM-treated membranes was not observed with phospholipase C. Thus, treatment of rabbit platelet membranes with NEM inhibited [^{3}H]IP_2 formation to similar degrees in the absence and presence of GTP, irrespective of whether NaCl or KCl was present. As with the treatment of rabbit platelet membranes with pertussis toxin, the inhibitory action of NEM on the basal enzyme activities makes it difficult to determine whether the G protein(s) that have been modified by this agent mediate the activation of phospholipase C by PAF. However, treatment of rabbit platelet membranes with NEM decreased [^{3}H]IP_2 formation caused by PAF in suspensions containing either NaCl or KCl. This

action of NEM could not be accounted for by the inhibitory action of NEM on basal phospholipase C activity since the percent stimulation of [^{3}H]IP_2 formation by either 2 nM or 1 μM PAF was decreased.

Treatment of rabbit platelet membranes with NEM decreased the inhibition of adenylate cyclase by PAF but never completely blocked the inhibitory action of high concentrations of this agonist (Table 4.6.). This effect of NEM was seen in rabbit platelet membrane suspensions containing either NaCl or KCl. In a single experiment, NEM decreased the inhibition of adenylate cyclase by 2 nM PAF from 61% to 21%, that by 2 units of thrombin/ml from 85% to 38% and that by 5 μM epinephrine from 79% to 37% (percentage inhibition of mean values from triplicate determinations $\pm$ S.E.Q.). Only at a high concentration of PAF (1 μM) was treatment of rabbit platelet membranes with NEM relatively ineffective in decreasing the inhibition of adenylate cyclase by this agonist (from 91% to 59%).

Conclusions. The results suggest that PAF may inhibit adenylate cyclase and activate phospholipase C by both NEM-sensitive and NEM-insensitive pathways. Since the coupling of PAF receptors to both of these effector systems was partially blocked by this agent, it is possible that G_i-related proteins can mediate both actions of PAF. Though it is difficult to eliminate effects attributable to incomplete alkylation of target proteins, the results are consistent

with evidence suggesting that the inhibition of adenylate cyclase and activation of phospholipase C can be mediated by mechanisms that are not inactivated by NEM.

4.3. *Discussion*

4.3.1. *Simultaneous assay of adenylate cyclase and phospholipase C: modulation by PAF, thrombin, epinephrine and GTP*

In the present study, a new combination assay was designed for the measurement of adenylate cyclase and phospholipase C activities under identical experimental conditions, using a single preparation of [^{3}H]inositol-labelled rabbit platelet membranes. This was accomplished by prelabelling rabbit platelets with [^{3}H]inositol and modifying previous methods for preparing rabbit platelet membranes (Hrbolich *et al.*, 1987; Chapter 3). [α-^{32}P]ATP was used as the substrate for adenylate cyclase instead of [^{3}H]ATP. In addition, changes to previous methods for measuring adenylate cyclase (Chapter 3) and phospholipase C (Hrbolich *et al.*, 1987) activities were made so that the assay conditions and incubation medium of the combined system were not unduly deleterious to the activity of either enzyme.

The major water soluble product of activation of rabbit platelet membrane phospholipase C by GTPγS or by agonists in the presence

of GTP was IP_2 in both the original (Hrbolich *et al.*, 1987) and combination assays. In the former, the [^{3}H]inositol phosphates formed after incubation for 10 min with GTPγS consisted of 10% [^{3}H]IP, 88% [^{3}H]IP_2 and 2% [^{3}H]IP_3 (Hrbolich *et al.*,1987). PIP_2 is thought to be the major substrate of receptor-mediated activation of phospholipase C in many cells and accumulation of IP_2 is often assumed to reflect the degradation of IP_3. However, experiments designed to determine the metabolic precursor of the [^{3}H]IP_2 released from [^{3}H]inositol-labelled platelet membranes showed that only 20% was derived from [^{3}H]PIP_2 and that the remainder was formed by the direct action of phospholipase C on [^{3}H]PIP (Hrbolich *et al.*, 1987). Thus, the accumulation of [^{3}H]IP_2 was considered a valid measurement of phospholipase C activity in the combination assay.

Rabbit platelet particulate fractions assayed in the combined system retained hormone-responsive, GTP-dependent adenylate cyclase and phospholipase C activities. Thus, it was possible to demonstrate that PAF and thrombin were potent agonists with respect to both effector systems, whereas epinephrine functioned solely as an inhibitor of adenylate cyclase activity. These findings are in agreement with previous studies on intact and permeabilized platelets. Thus, thrombin has been shown to activate phospholipase C in intact platelets of various species (Rittenhouse-Simmons, 1979: Lapetina, 1983), as well as in permeabilized human platelets (Haslam

and Davidson, 1984a,b,c; Culty *et al.*, 1988). Thrombin also decreases cAMP formation in intact and permeabilized human platelets (Mills, 1974; Brass *et al.*, 1988) and inhibits adenylate cyclase in human platelet membranes (Aktories and Jakobs, 1984). Similarly, it has been found that PAF activates phospholipase C in intact human and rabbit platelets (Siess *et al.*, 1984; Shukla, 1985), as well as in permeabilized human platelets (Haslam *et al.*, 1985), and that PAF decreases cAMP formation caused by PGE_1 in rabbit platelets (Haslam and Vanderwel, 1982). PAF also inhibits adenylate cyclase activity in platelet membranes from both species (Chapter 3). Although epinephrine inhibits adenylate cyclase in human platelet membranes (Jakobs *et al.*, 1978), alone this compound has no effect on phosphoinositide metabolism in human platelets (Siess *et al.*, 1984). Moreover, in contrast to the actions of other stimulatory platelet agonists, such as thrombin, PAF, vasopressin and U46619, epinephrine does not decrease the requirement of [^{14}C]serotonin secretion for Ca^{2+} in permeabilized platelets (Knight and Scrutton, 1985). However, platelet aggregation, secretion of [^{14}C]serotonin and Ca^{2+} mobilization caused by a number of stimulatory platelet agonists are potentiated by epinephrine (Cameron and Ardlie, 1982; Vargaftig *et al.*, 1982; Bushfield *et al.*, 1987), though this effect does not appear to be due to α_2-receptor-mediated inhibition of adenylate cyclase (Thompson *et al.*, 1986). Epinephrine also potentiated the actions of PAF and thrombin on phospholipase C activity in the

present study and that of ADP in rabbit platelets (Lalau Keraly *et al.*, 1987) and of thrombin in human platelets (Steen *et al.*, 1988). Since the ability of epinephrine to enhance the action of other platelet agonists was observed in platelet membranes, it cannot be attributed to an indirect effect of a second messenger in this study.

Measurement of adenylate cyclase and phospholipase C activities under the same conditions demonstrated that optimal activation of phospholipase C by PAF required much higher concentrations of the agonist and was more highly dependent on GTP than was the inhibition of adenylate cyclase by this compound. Thus, inhibition of adenylate cyclase and activation of phospholipase C by PAF could be mediated by a single receptor type with different affinities for PAF as a result of interaction with distinct G proteins. Alternatively, if a single receptor with a single agonist affinity is involved, optimal activation of phospholipase C by PAF could require a larger receptor occupancy than inhibition of adenylate cyclase. However, distinct receptor types and/or distinct G proteins coupled to adenylate cyclase and phospholipase C could also account for the differences in agonist and GTP concentrations required by these two effector enzymes.

These findings in platelets are reminiscent of the much greater potency and efficacy of muscarinic agonists in inhibiting

adenylate cyclase than in activating phospholipase C in embryonic chick heart cells (Heller-Brown and Brown, 1984) and in chinese hamster ovary cells containing transfected M2 muscarinic cholinergic receptors (Ashkenazi *et al.*, 1987). It is unlikely that differences observed by Ashkenazi *et al.* (1987) were experimental artifacts because both enzymes were monitored under the same conditions and similar ED_{50}s for thrombin were observed for both enzyme systems. Thus, in some cells in which the receptors involved are more clearly defined than in platelets, higher concentrations of agonist are required for optimal activation of phospholipase C than for inhibition of adenylate cyclase. Assuming that a single receptor type mediates both effects, there may be spare receptors for the inhibition of adenylate cyclase that must be occupied for optimal activation of phospholipase C. In support of this hypothesis is the observation that the dose-response curve for activation of phospholipase C by carbachol in chick heart cells lies to the right of that for inhibition of adenylate cyclase and is very similar to the binding isotherm for this agonist (Heller Brown and Brown, 1984).

4.3.2. *Role of G_i in phospholipase C activation*

To determine whether G_i was involved in the activation of phospholipase C by PAF, rabbit platelet membranes were incubated with pertussis toxin prior to the measurement of the effects of PAF on

[^{32}P]cAMP and [^{3}H]IP_2 formation. Treatment of rabbit platelet membranes with the pertussis holotoxin or purified S_1 subunit decreased both the inhibition of adenylate cyclase by PAF and the absolute amounts of [^{3}H]IP_2 formed in the absence and presence of PAF and GTP. However, the toxin had no effect on the percent stimulation of [^{3}H]IP_2 formation caused by PAF. Although these findings suggest that G_i does not mediate more than a part of the activation of phospholipase C by PAF, definite proof of this could not be obtained. Thus, as the ADP-ribosylation of the pertussis toxin substrates was incomplete, it is possible in principle that the small percentage that was not inactivated by the toxin could couple a disproportionate number of PAF receptors to phospholipase C. However, against this it could be argued that since activation of phospholipase C by PAF probably requires occupation of more receptors than inhibition of adenylate cyclase, it is likely that activation of phospholipase C also requires more G protein. Also, the inhibition of adenylate cyclase by PAF was reduced but never completely blocked by either the holotoxin or the S_1 subunit. Since inhibition of adenylate cyclase by PAF still remained in rabbit platelet membranes in which pertussis toxin had almost completely abolished the action of epinephrine, it appears that PAF inhibits adenylate cyclase by both pertussis toxin-sensitive and insensitive pathways. It was difficult to interpret pertussis toxin-induced changes in [^{32}P]cAMP and [^{3}H]IP_2 formation in samples incubated with PAF because the toxin treatment decreased basal adenylate cyclase and phospholipase C

activities. Since stimulation of adenylate cyclase by Na^+ occurs by a GTP-dependent mechanism (Chapter 3), a decrease in basal enzyme activity by pertussis toxin suggests that this effect of Na^+ may be mediated by a pertussis toxin substrate. However, comparison of the inhibitory actions of the holotoxin and the S_1 subunit on basal enzyme activities with the extent of ADP-ribosylation of pertussis toxin substrates demonstrated that the decreases in [^{32}P]cAMP and [^{3}H]IP_2 formation caused by the holotoxin could not be accounted for solely by the incorporation of ADP-ribose into the pertussis toxin substrates. In contrast to these unexplained inhibitory effects of the holotoxin observed with rabbit platelet membranes, the holotoxin has been reported to aggregate intact human platelets as a result of an ability of the non-catalytic subunits to activate phospholipase C (Banga *et al.*, 1987).

Studies in other laboratories designed to determine whether G_i couples Ca^{2+}-mobilizing receptors to phospholipase C using human permeabilized platelets and platelet membrane preparations have given contradictory results. Lapetina (1986) reported that ADP-ribosylation of a 42 kDa protein that co-migrated with the α_i-subunit enhanced the stimulatory action of thrombin on phospholipase C in human platelets permeabilized with saponin. This finding may reflect the ability of the holotoxin to activate phospholipase C (Banga *et al.*, 1987). Support for a role for pertussis toxin-sensitive G-protein in the activation of

phospholipase C by some agonists but not others, comes from the studies of Brass *et al.* (1986; 1987) using saponin-permeabilized human platelets. These workers found that both thrombin and U46619-induced phosphoinositide hydrolysis was attenuated by GDPβS but only the action of thrombin was blocked by pertussis toxin. O'Rourke *et al.* (1987) have also reported that pertussis toxin blocks the release of Ca^{2+} from platelet dense tubular vesicles incubated with platelet plasma membranes, thrombin and GTP. The latter workers observed two pertussis toxin substrates with molecular masses of 39 and 41 kDa by SDS-polyacrylamide gel electrophoresis in human platelet membranes (O'Rourke *et al.*, 1987), whereas Brass *et al.* (1988) were only able to resolve two species by isoelectric focusing (both 41 kDa). In an attempt to determine which receptors and effectors were coupled to these G_i-like proteins, Brass *et al.* (1988) examined the effects of agonists that inhibit adenylate cyclase and/or stimulate phosphoinositide breakdown on their [^{32}P]ADP-ribosylation by pertussis toxin in permeabilized platelets. [^{32}P]ADP-ribosylation of both G proteins was decreased to the same extent (50%) by agonists that specifically activated phospholipase C in a pertussis toxin-sensitive manner (PAF and vasopressin) or inhibited adenylate cyclase (epinephrine) in the permeabilized human platelets. Incubation of human platelets with thrombin, which both activated phospholipase C and inhibited adenylate cyclase, blocked [^{32}P]ADP-ribosylation of both 41 kDa proteins by greater than 90%.

No effect was observed with U46619, which correlated with the pertussis toxin-insensitive activation of phospholipase C caused by this compound and its inability to inhibit platelet adenylate cyclase activity in permeabilized platelets. Thus, by using decreased susceptibility to [^{32}P]ADP-ribosylation by pertussis toxin as an index of the amount of G protein bound by specific hormone-receptor complexes, Brass *et al.* (1988) concluded that they had shown that agonist-induced activation of phospholipase C and inhibition of adenylate cyclase were mediated by the same G proteins in human platelets. It appeared that two variants of a single pertussis toxin substrate with identical peptide maps were involved in both signal transduction mechanisms. However, a larger percentage of these G protein subspecies were involved when the agonist both stimulated phospholipase C and inhibited adenylate cyclase. In contrast, the presence of distinct G proteins mediating inhibition of adenylate cyclase and stimulation of phospholipase C by agonists is supported by a recent study by Banga *et al.* (1988) demonstrating different susceptibilities of the two enzyme systems to the S_1 subunit of pertussis toxin. Treatment of human platelets permeabilized with saponin with the S_1 subunit of pertussis toxin blocked decreases in PGE_1-stimulated cAMP accumulation by thrombin but had very little effect on PA formation caused by this agonist. These findings agree with those obtained using rabbit platelet membranes in the present thesis.

Indirect evidence suggesting a role for pertussis toxin-insensitive G protein in activation of phospholipase C in human platelets comes from the finding that pertussis toxin only partially inhibited the GTPase activities stimulated by thrombin, U44069 and PAF but completely blocked epinephrine-stimulated GTPase under the same conditions (Houslay *et al.*, 1986a,b; Grandt *et al.*, 1986). Since enzyme activity remained after pertussis toxin treatment only in samples containing agonists which activate phospholipase C, the GTPase activity was thought to reflect the activity of the G protein coupled to this enzyme. Also, pertussis toxin treatment blocked the inhibition of adenylate cyclase by thrombin and epinephrine in membranes preparations in which it only partially inhibited the GTPase activity stimulated by thrombin and had no effect on vasopressin-induced GTPase activity (Grandt *et al.*, 1986). Since α_2-adrenergic receptors coupled to G_i did not stimulate phospholipase C activity and the vasopressin-induced GTPase activity was additive with that of epinephrine, it was thought unlikely that G_i mediates activation of phospholipase C in human platelets (Houslay *et al.*, 1986a; Grandt *et al.*, 1986). It should be noted, however, that pertussis-insensitive GTPase could also reflect activation of G proteins linked to effectors other than or additional to phospholipase C.

Since treatment of human platelet membranes with the thiol reagent, NEM, has been shown to block the inhibition of adenylate cyclase by thrombin (Aktories and Jakobs, 1984) and epinephrine (Jakobs *et al.*, 1982; Limbird and Speck, 1983), while leaving stimulation of adenylate cyclase intact, this agent has been used to determine the role of G_i in coupling receptors to other effectors. NEM has also been shown to alkylate the cysteine residue in GTP-binding proteins that incorporates ADP-ribose upon incubation with pertussis toxin (Ui and Katada, 1987). Since NEM and pertussis toxin selectively inactivate G_i-like proteins, an attempt was made to determine the role of G_i-related proteins in phospholipase C activation by treating rabbit platelet membranes with NEM prior to the measurement of PAF-induced [^{3}H]IP_2 formation in the combination system. In agreement with the action of pertussis toxin and with previous studies using other agonists, NEM decreased the inhibition of adenylate cyclase by PAF. However, the effects of concentrations of PAF giving optimal inhibition of adenylate cyclase were not completely blocked by the NEM concentrations used, suggesting that an NEM-insensitive pathway may exist for inhibition of adenylate cyclase by PAF in rabbit platelet membranes. Similarly, in NG108-15 neuroblastoma x glioma cells, the inhibition of adenylate cyclase by epinephrine and the guanine-nucleotide sensitivity of the high affinity epinephrine binding were much more resistant to the action of NEM than were the corresponding actions of muscarinic and opiate receptors (Smith and Harden, 1984).

Preincubation of rabbit platelet membranes with NEM also inhibited basal adenylate cyclase and phospholipase C activities. Since the inhibitory action of NEM on adenylate cyclase was greater in membrane suspensions containing NaCl than KCl, it is likely that NEM acts to inhibit the stimulatory action of NaCl on basal enzyme activity. However, as for the action of pertussis toxin on the basal enzyme activities, these inhibitory effects of NEM made it difficult to determine whether reduced agonist effects in treated rabbit platelet membranes resulted from inactivation of G_i or direct enzyme inhibition. Inhibition of forskolin-stimulated adenylate cyclase by NEM suggests that this agent may not only inactivate G_i but may also act on the catalytic unit of this enzyme (Aktories *et al.*, 1982b).

In contrast to the similar effects of pertussis toxin and NEM on adenylate cyclase discussed above, NEM treatment of rabbit platelet membranes significantly reduced the percent stimulation of $[^3H]IP_2$ formation caused by PAF in the combination system. It is possible that NEM inactivates a G protein involved in phosphoinositide metabolism that is insensitive to pertussis toxin or has direct effects on PAF receptors themselves.

4.3.3. *Monovalent cation effects on adenylate cyclase and phospholipase C*

To determine whether the activities of adenylate cyclase and phospholipase C observed in the absence and presence of PAF are affected similarly by monovalent cations, rabbit platelet particulate fractions were assayed in medium containing 100 mM NaCl or 100 mM KCl. In agreement with previous studies (Chapter 3), basal adenylate cyclase activity was greater in the presence of NaCl than KCl. This action of NaCl, which may be partly mediated by G_i (Chapter 3; Aktories *et al.*, 1982b), was substantially reduced in NEM-treated rabbit platelet particulate fractions. The stimulatory action of NaCl on adenylate cyclase was also inhibited in NEM-treated adipocyte ghosts (Aktories *et al.*, 1982b). Although, there is evidence to support a Na^+ site on G_i, the ability of NEM to block guanine nucleotide-induced decreases in α_2-adrenergic receptor affinity for agonists without interfering with Na^+-induced decreases in affinity suggests that Na^+ and GTP can act separately through different components of the system (Limbird and Speck, 1983). In contrast to the greater stimulatory action of NaCl than KCl on adenylate cyclase activity, [^{3}H]IP_2 formation was greater in the presence of KCl than NaCl. This suggests either that G_i is not involved in the activation of phospholipase C or that Na^+ acts primarily on a component other that G_i. In support of the latter hypothesis are the findings of Ott *et al.* (1988) that a

Na^+-sensitive protein distinct from a G protein accounted for the difference in the molecular sizes of the opioid receptor as determined by target size analysis and SDS polyacrylamide gel electrophoresis.

4.3.4. *Pertussis toxin substrates*

Studies designed to characterize the pertussis toxin substrates in a number of cells have uncovered more than one substrate in bovine (Neer *et al*, 1984) and porcine brain (Katada *et al.*, 1987), rat fat cells, chick myocytes (Rapiejko *et al.*, 1986), bovine (Mumby *et al.*, 1986) and rabbit (Malbon *et al.*, 1985) heart preparations, anterior pituitary cells (Journet *et al.*, 1987), myeloid HL60 cells (Uhing *et al.*, 1987) and now human (O'Rourke *et al*, 1987) and rabbit platelets. In bovine brain (Neer *et al.*, 1984) and anterior pituitary cells (Journet *et al*, 1987), there are three polypeptides (molecular masses 41, 40 and 39 kDa) that are [^{32}P]ADP-ribosylated by pertussis toxin. However, these G proteins may not be equally susceptible to pertussis toxin. In bovine brain, the 41 kDa protein, which co-migrates with the α_i, has a much greater affinity for the $\beta\gamma$-subunits and is therefore much more readily ADP-ribosylated by pertussis toxin than the 40 kDa and 39 kDa proteins (Huff and Neer, 1986). In contrast, the 40 kDa protein is more readily labelled in anterior pituitary cells (Journet *et al.*, 1987) and in myeloid HL60

membranes, the 40 kDa and 41 kDa proteins are [^{32}P]ADP-ribosylated to the same extent (Uhing *et al.*, 1987). This disparity in the extent of [^{32}P]ADP-ribosylation of pertussis toxin substrates could reflect differences in susceptibility to pertussis toxin or to a difference in amounts of the pertussis toxin substrates in these cells.

In the present study, three pertussis toxin substrates of 41 kDa, 40/39 kDa and 38 kDa have been consistently identified in rabbit platelet membranes. The smaller species do not appear to be proteolytic fragments of G_i. The pattern of [^{32}P]ADP-ribosylation was very similar to that reported in bovine cerebral cortex (Neer *et al.*, 1984) with the 41 kDa polypeptide being the most heavily labelled. Thus, it appears that an increasing number of cells including platelets contain a heterogeneous mixture of G_i-related proteins present in variable amounts in different cells and tissues. The two lower molecular mass pertussis toxin substrates found in rabbit platelet membranes could be the 40 kDa and 39 kDa proteins identified in other cell membranes.

A 40 kDa pertussis toxin substrate has been identified in bovine (Neer *et al.*, 1984) and porcine (Katada *et al.*,1987) brain , NG108-15 hybrid neuroblastoma-glioma cells (Milligan *et al.*, 1986), C_6 glioma cells (Milligan *et al.*, 1986), chromaffin granules (Toutant *et al.*, 1987), HL60 cells (Gierschik

and Jakobs, 1987), neutrophils (Becker *et al.*, 1987), rabbit liver, rat erythrocyte and mouse S49 lymphoma cell membranes (Mumby *et al.*, 1988). It has been possible to separate the 40 kDa protein from the α_i and α_o subunits in bovine and porcine brain as well as from α_i in HL60 cell membranes (Uhing *et al.*, 1987) and to show immunologically and biochemically that the 40 kDa protein is distinct from α_i, α_o and α_T (Mumby *et al.*, 1988; Becker *et al.*, 1987; Uhing *et al.*, 1987). A 40 kDa GTP-binding coupled to the fMLP receptor in HL60 membranes is a substrate for cholera toxin and [^{32}P]ADP-ribosylation is increased by fMLP and blocked by pretreatment with pertussis toxin (Gierschik and Jakobs, 1987). Since fMLP receptors are coupled to phospholipase C but not to adenylate cyclase and cholera toxin has been reported to block phosphoinositide metabolism by a cAMP-independent mechanism (Imboden *et al.*, 1986; Lo and Hughes, 1987), the 40 kDa protein is thought to be the G protein involved in phospholipase C activation by fMLP in neutrophils (Becker *et al.*, 1987) and HL60 cells (Gierschik and Jakobs, 1987).

G proteins of 39 kDa detected by their incorporation of [^{32}P]ADP-ribose upon exposure to pertussis toxin, have been found in bovine retinal rods (G_T) (Van Dop *et al.*, 1984), rat fat cells (Rapiejko *et al.*, 1986), chick myocytes (Rapiejko *et al.*, 1986), bovine (Mumby *et al.*, 1986) and rabbit (Malbon *et al.*, 1985) heart preparations and human platelets (O'Rourke *et*

al., 1987). cDNA sequencing and immunochemical data show that besides α_{41} and α_{40} and α_{39}, there is another G_i-like protein in a number of cell types which is a substrate for pertussis toxin (Jones and Reed, 1987; Gierschik *et al.*, 1986). Nagata *et al.* (1988) have recently purified two pertussis toxin substrates from human platelet membranes and shown that the major substrate of 40 kDa cross-reacts with $G_{i2\alpha}$-specific antibodies. The minor pertussis toxin substrate of 41 kDa cross-reacts with $G_{i1\alpha}$-specific antibodies.

Thus, in the earlier studies where the majority of cells including platelets were shown to contain only a single pertussis toxin substrate, it is possible that the other lower molecular weight substrates were present but not resolved. As a result of the increasing number of pertussis toxin substrates that are being found, it is no longer possible to attribute the mediation of all pertussis toxin-sensitive processes to the G protein thought to be involved in hormonal inhibition of adenylate cyclase (G_i). It is possible that even in pertussis toxin-sensitive cells, distinct G proteins may couple receptors to adenylate cyclase and phospholipase C. Alternatively G proteins may be able to interact with a number of receptors and effectors, the precise combination depending on the experimental conditions. Non-specificity of G protein action is supported by the recoupling of fMLP-stimulated phospholipase C in pertussis toxin-treated HL60 cells by either purified G_i or G_o

(Kikuchi *et al.*, 1986). However, the evidence to date, suggests that the 40 kDa G_i-related protein is the most likely candidate in the latter cells.

4.3.5. *Possible candidates for the G protein mediating pertussis toxin-insensitive activation of phospholipase C*

Since the activation of phospholipase C is pertussis toxin-insensitive in a number of cells (Uhing *et al.*, 1986; Masters *et al.*, 1985; Hepler and Harden, 1986) and in human platelets under certain conditions (Lapetina, 1986a; Brass *et al.*, 1987; this thesis), it is likely that GTP-binding proteins that are not substrates of pertussis toxin can couple Ca^{2+}-mobilizing receptors to this enzyme. Possible candidates include the p21 ras gene products (Wakelam *et al.*, 1986; Fleishman *et al.*, 1986), a 21-25 kDa protein ($G_p/G_{\alpha 25}$) isolated from placenta, platelets and brain that does not cross-react with monoclonal antibody to ras (Evans *et al.*, 1986; Waldo *et al.*, 1987), ADP-ribosylation factor (Kahn and Gilman, 1986), botulinum toxin substrates (Ohashi *et al.*, 1987; Aktories and Frevert, 1987; Rosener *et al.*, 1987; Aktories *et al.*, 1987), $G_{z\alpha}$ (Fong *et al.*, 1988) and other low molecular mass GTP-binding proteins (G_n) (approx. 23-27 kDa) identified on nitrocellulose blots of membrane proteins from rabbit and human platelets (Bhullar and Haslam, 1987; Lapetina and Reep, 1987) and

human neutrophils (Bokoch and Parkos, 1988). The G_n-proteins were detected by high affinity binding of [α-^{32}P]GTP to proteins on nitrocellulose blots after their separation under denaturing conditions by SDS-polyacrylamide gel electrophoresis, and are distinct from ras proteins, botulinum toxin substrates and $G_p/G_{\alpha 25}$ (Bhullar and Haslam, 1988). These proteins are trypsin resistant and have been postulated to mediate trypsin-resistant activation of phospholipase C by GTPγS in human platelets (Lapetina and Reep, 1987). They are not pertussis or cholera toxin substrates (Bokoch and Parkos, 1988). Low molecular weight GTP-binding proteins have also been identified in rat liver membranes (25 kDa; Heyworth *et al.*, 1985), bovine brain (25 kDa; Waldo *et al.*, 1987; 24kDa; Kikuchi *et al.*, 1988) and HL60 cell membranes (23 kDa; Uhing *et al.*, 1987). although the G_n proteins, ras p21 proteins, botulinum toxin substrates and $G_p/G_{\alpha 25}$ are all candidates for mediation of pertussis toxin-insensitive activation of phospholipase C in platelets, the only direct evidence yet available favours the first of these. Baldassare *et al.* (1988) demonstrated that a soluble G protein of 29 kDa (apparently identical to G_n) co-chromatographed with platelet phospholipase C and reconstituted GTPγS-stimulated enzyme activity.

4.4. *Summary*

4.4.1. The effects of PAF, thrombin and epinephrine on the adenylate cyclase and phospholipase C activities present in the same rabbit platelet membrane preparations were examined under identical experimental conditions by measuring the formation of [^{32}P]cAMP from [α-^{32}P]ATP and the release of [^{3}H]IP_2 from endogenous [^{3}H]polyphosphoinositides. PAF and thrombin were potent stimulators of phospholipase C and inhibitors of adenylate cyclase, whereas epinephrine acted solely as an inhibitor of adenylate cyclase.

4.4.2. Addition of GTP enhanced both the activation of phospholipase C and the inhibition of adenylate cyclase by PAF. However, optimal activation of phospholipase C required higher concentrations of PAF and was more highly dependent on added GTP than was inhibition of adenylate cyclase.

4.4.3. Pertussis toxin ADP-ribosylated three proteins in the 38-42 kDa range in rabbit platelet particulate fractions. Incubation of these particulate fractions with activated pertussis holotoxin or its S_1 subunit decreased basal adenylate cyclase and phospholipase C activities and reduced but did not completely block the inhibitory action of PAF plus GTP and of epinephrine plus GTP on adenylate cyclase activity. However, no effect of pertussis toxin was observed on the percent stimulation of

$[^3H]IP_2$ formation caused by PAF, suggesting that activation of phospholipase C was partly mediated by a pertussis toxin-insensitive G protein. Since pertussis toxin decreased the inhibition of adenylate cyclase by PAF less effectively than it did the inhibition caused by epinephrine, both pertussis toxin-sensitive and -insensitive pathways may exist for inhibition of the enzyme by this agonist.

4.4.4. Incubation of rabbit platelet particulate fractions with NEM decreased basal adenylate cyclase and phospholipase C activities and reduced but did not completely block the percent inhibition of adenylate cyclase by PAF, thrombin and epinephrine. In contrast to the findings with pertussis toxin, NEM treatment decreased the percent stimulation of $[^3H]IP_2$ formation caused by PAF. A NEM-sensitive, pertussis toxin-insensitive pathway for stimulation of phospholipase C may exist in rabbit platelets.

4.4.5. The results can only be explained by the involvement of multiple receptors and/or G proteins in the actions of PAF on the effectors studied.

Fig. 4.1. Effects of increasing concentrations of PAF on rabbit platelet adenylate cyclase and phospholipase C activities in the absence and presence of GTP

Membranes from platelets labelled with [^{3}H]inositol were washed twice with hypotonic buffer before final resuspension (Section 2.2.9.). Samples containing 0.148 mg of protein were then assayed for adenylate cyclase and phospholipase C activities in 10 min incubations at 25°C, as described in Section 2.2.10. Assays were carried out with 0 - 1 μM PAF in the absence (open symbols) and presence (closed symbols) of 10 μM GTP. [^{3}H]IP_2 (●, ○) and [^{32}P]cAMP (▲, △) were isolated and counted and the amounts present before incubation were subtracted. Values are means from triplicate incubation mixtures. Error bars are not shown because the S.E.M. lies within the areas of the symbols.

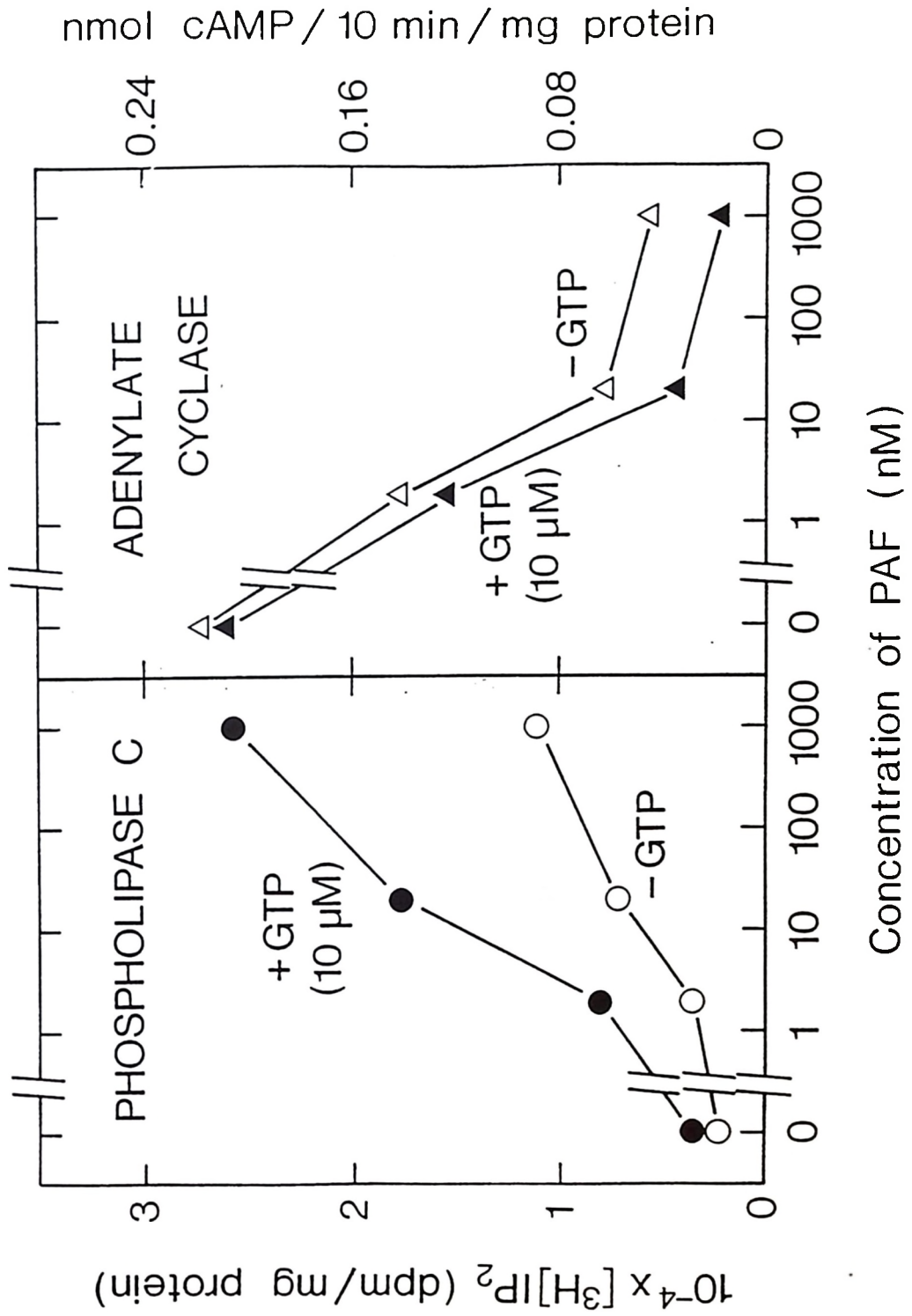

nmol cAMP / 10 min / mg protein
0.24
0.16
0.08
0
ADENYLATE CYCLASE
+GTP (10 μM)
-GTP
PHOSPHOLIPASE C
+GTP (10 μM)
-GTP
3
2
1
0
0 1 10 100 1000 0 1 10 100 1000
Concentration of PAF (nM)
10⁻⁴ x [³H]IP₂ (dpm/mg protein)

Fig. 4.2. [^{32}P]ADP ribosylation of proteins in rabbit platelet membranes by pertussis toxin

Rabbit platelet particulate fraction prepared as in 2.2.9. was incubated with NAD^+ and pertussis toxin and the extent of ADP-ribosylation of platelet substrates determined (Section 2.2.13.). The platelet membranes were first incubated with 1 mM NAD^+ in the absence or presence of pertussis toxin and then, after washing, with 10 μM [^{32}P]NAD^+ and pertussis toxin. Both incubations were for 90 min at 30°C; 12 μg of activated pertussis holotoxin/ml was used. Protein (50 μg) was analyzed by SDS-polyacrylamide gel electrophoresis. An audioradiograph of the dried gel was obtained (see opposite) before excision of the labelled protein for counting. Lanes were as follows: (a) no pertussis toxin present during the first incubation; (b) pertussis toxin present during the first incubation. Calculation indicated that pertussis toxin ADP-ribosylated 62% of the pertussis toxin substrates during the incubation with unlabelled NAD^+.

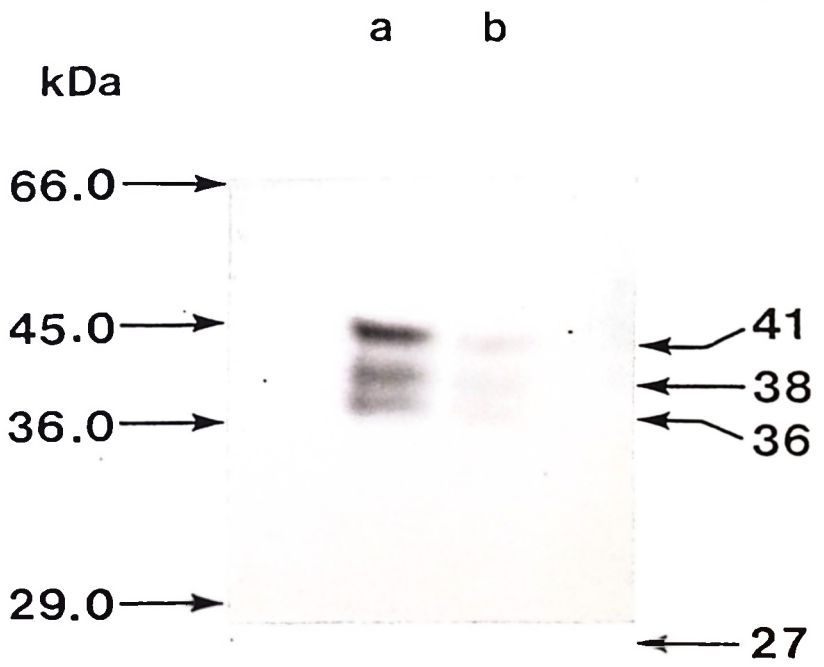


Fig. 4.3. [^{32}P]ADP-ribosylation of platelet proteins by pertussis toxin in rabbit platelet membranes prepared and incubated in the absence and presence of proteolytic inhibitors

Washed rabbit platelets were resuspended at 25 mg/ml in 150 mM Tris-HCl (pH 7.4) containing either (a) no other additions, (b) EGTA (5 mM) or (c) EGTA (5 mM), leupeptin (200 μg/ml), PMSF (1 mM), benzamidine (1 mM) and soybean trypsin inhibitor (100 μg/ml). Platelet particulate fractions were prepared by freezing and thawing the suspensions and centrifugation, and were then incubated with 20 μg of pertussis toxin/ml and 10 μM [^{32}P]NAD for 90 min at 30^{o}C (see Section 2.2.13.). The platelet membranes were washed twice prior to precipitation of protein with trichloroacetic acid (10% w/v). Protein (50 μg) was analysed by SDS-polyacrylamide gel electrophoresis and an audioradiograph of the dried gel is shown. Lanes are as follows: (a) protein from platelets lysed in 150 mM Tris-HCl, pH 7.4, with no other additions, (b) protein from platelets lysed in buffer containing EGTA and (c) protein from platelets lysed in buffer containing EGTA, leupeptin, PMSF, benzamidine and soybean trypsin inhibitor. Proteolytic inhibitors were included in the incubation mixture used for ADP-ribosylation of platelet membranes in lane c.

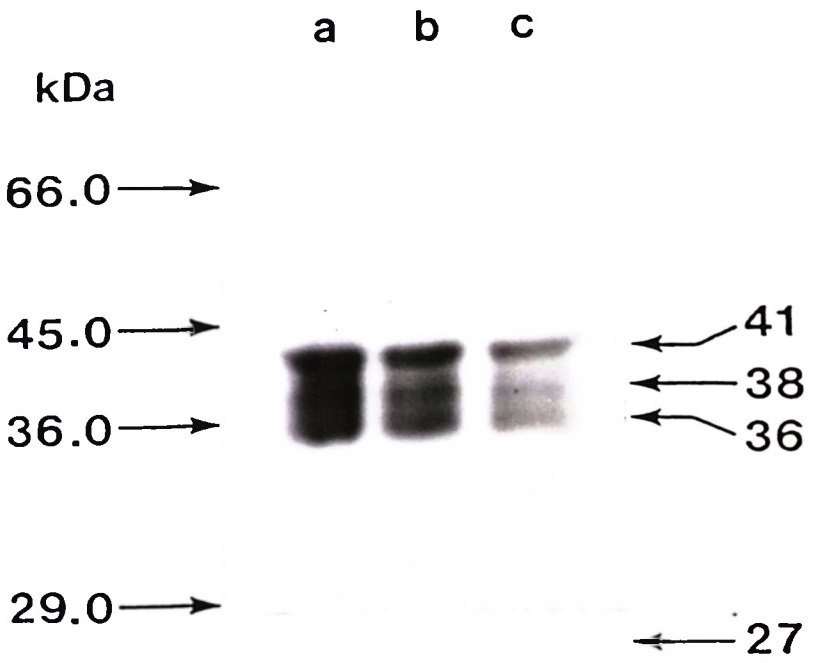


Table 4.1. Assay conditions for measurement of platelet membrane adenylate cyclase and phospholipase C activities

Membranes from rabbit and human platelets were assayed for adenylate cyclase alone (Chapter 3) in the presence of the additions listed on the left (see also Section 2.2.8.). The assay conditions used by Hrbolich *et al.* (1987) for measurement of phospholipase C in rabbit platelet membranes are also given (centre). Finally, the modifications to these two methods used for measurement of both adenylate cyclase and phospholipase C activities in the combination system are also shown on the right (see also Section 2.2.10.)

4.1.

	Assay		
	Adenylate cyclase	Phospholipase C	Combination
Membranes			
Label	None	[^{3}H]Inositol	[^{3}H]Inositol
Protein (µg)	100-150	200-300	100-150
Additions			
Tris-HCl, pH 7.4 (mM)	75.0	-	-
Hepes, pH 7.4 (mM)	-	25.0	25.0
EGTA (mM)	0.4	2.5	2.5
NaCl (mM)	0-150	-	100
KCl (mM)	-	100	-
ATP (mM)	0.4	1.23	0.46
[^{3}H]ATP (µCi)	2.0	-	-
[^{32}P]ATP (µCi)	-	-	2.0
$MgCl_2$ (mM)	5.0	1.56	0.96
$CaCl_2$ (mM)	-	1.48	1.48
IBMX (mM)	1.0	-	1.0
CP (mM)	4.0	-	4.0
CPK (units/ml)	20.0	-	20.0
DTT (mM)	0.4	-	0.4
cAMP (mM)	1.0	-	-
BSA (mg/ml)	1.0	-	1.0
Assay conditions			
Temperature (^{o}C)	30	25	25
Incubation period (min)	10	10	10
Volume (ml)	0.25	0.5	0.25

Table 4.2. Effects of different $MgCl_2$ concentrations and of cAMP on the adenylate cyclase and phospholipase C activities of platelet membranes measured in the absence and presence of PAF and GTP

Membranes from platelets labelled with [^{3}H]inositol were washed twice with hypotonic buffer before final resuspension (Section 2.2.9.). Samples containing 190 µg of membrane protein were assayed for adenylate cyclase and phospholipase C activities in 10 min incubations at 25°C, as described in Section 2.2.10. [α-^{32}P]ATP (2 µCi) was included in those samples from which [^{32}P]cAMP was to be isolated. Final concentrations of the additions were 1 mM cAMP, 200 nM PAF and 10 µM GTP, as indicated. [^{32}P]cAMP, [^{3}H]IP_1, [^{3}H]IP_2 and [^{3}H]IP_3 were isolated and counted and the amounts present before incubation were subtracted. Values for [^{32}P]cAMP and [^{3}H]IP_2 formed in triplicate incubation mixtures are given as means ± S.E.M. and means ± S.E.D., respectively.

4.2.

$MgCl_2$ (mM)	Additions	Adenylate cyclase activity	Phospholipase C activity		
		$[^{32}P]$cAMP formed (nmol/10 min/ mg protein)	$[^3H]$Inositol phosphates formed (d.p.m./10 min/mg protein)		
			$[^3H]IP_1$	$[^3H]IP_2$	$[^3H]IP_3$
0.96	None	0.435 ± 0.010	-21 ± 111	1,726 ± 179	26 ± 63
	PAF + GTP	0.041 ± 0.001	579 ± 126	10,311 ± 505	163 ± 74
	cAMP	0.430 ± 0.007	-68 ± 184	1,984 ± 137	116 ± 126
	cAMP+ PAF + GTP	0.037 ± 0.001	621 ± 158	9,021 ± 579	84 ± 63
4.8	None	1.189 ± 0.035	79 ± 116	1,447 ± 89	95 ± 74
	PAF + GTP	0.101 ± 0.002	2,179 ± 100	7,989 ± 416	242 ± 105
	cAMP	1.228 ± 0.009	-184 ± 111	974 ± 189	26 ± 74
	cAMP +PAF + GTP	0.089 ± 0.001	1,263 ± 153	6,005 ± 284	226 ± 74

Table 4.3. Effects of GTP, PAF, thrombin and epinephrine on the adenylate cyclase and phospholipase C activities of rabbit platelet particulate fractions

Membranes from platelets labelled with [^{3}H]inositol were washed twice with hypotonic buffer before final resuspension (Section 2.2.9.). Samples containing 148 µg of membrane protein were assayed for adenylate cyclase and phospholipase C activities in 10 min incubations at 25°C, as described in Section 2.2.10. [α-^{32}P]ATP (2 µCi) was included in those samples from which [^{32}P]cAMP was to be isolated. The final concentration of GTP was 10 µM. Epinephrine (5 µM) was added with 10 µM propranolol. Other additions were at the concentrations indicated. [^{3}H]IP_2 and [^{32}P]cAMP were isolated and counted and the amounts present before incubation were subtracted. Values for [^{32}P]cAMP and [^{3}H]IP_2 formed in triplicate incubation mixtures are given as means ± S.E.M. and means ± S.E.D., respectively. Both the percent inhibition of adenylate cyclase (± S.E.Q.) and percent stimulation of phospholipase C (± S.E.Q.) by agonists were calculated with respect to the values in incubation mixtures containing GTP alone.

4.3.

Additions	Adenylate cyclase activity		Phospholipase C activity	
	$[^{32}P]$cAMP formed (nmol/10 min/ mg protein)	Inhibition with respect to GTP control (%)	$[^{3}H]IP_2$ formed (dpm/10 min/ mg protein)	Stimulation with respect to GTP control (%)
None	0.219 ± 0.015	-	2,250 ± 223	-
GTP	0.209 ± 0.009	-	3,412 ± 176	-
EPI + GTP	0.057 ± 0.002	73 ± 2	3,851 ± 486	13 ± 15
Thrombin (0.2 units/ml) + GTP	0.092 ± 0.043	56 ± 21	17,446 ± 1493	411 ± 51
Thrombin (0.2 units/ml) + GTP + EPI	0.022 ± .004	89 ± 2	21,608 ± 953	533 ± 43
Thrombin (2.0 units/ml) + GTP	0.033 ± 0.002	84 ± 1	24,351 ± 236	614 ± 37
PAF (20 nM) + GTP	0.035 ± 0.001	83 ± 1	17,831 ± 743	423 ± 35
PAF (20 nM) + EPI + GTP	0.022 ± 0.002	89 ± 1	23,703 ± 284	596 ± 37
PAF (1 μM) + GTP	0.018 ± 0.001	91 ± 1	25,939 ± 446	660 ± 41

Table 4.4. Effects of [^{32}P]ADP-ribosylation of membrane proteins by pertussis toxin on rabbit platelet membrane adenylate cyclase and phospholipase C activities

Membranes from platelets labelled with [^{3}H]inositol as described in Section 2.2.9. were incubated for 30 min at 37°C in the absence or presence of activated pertussis holotoxin and the extent of [^{32}P]ADP-ribosvlation of platelet proteins was determined (Section 2.2.13). The membranes were washed twice by centrifugation and homogenization with hypotonic buffer before final resuspension (Section 2.2.9.). Samples containing 0.1-0.2 mg of membrane protein were assayed for adenylate cyclase and phospholipase C activities in 10 min incubations at 25°C, as described in Section 2.2.10. [α^{32}P]ATP (2 μCi) was included in those samples from which [^{32}P]cAMP was to be isolated. Final concentrations of the additions were 1 μM PAF, 5 μM epinephrine (with 10 μM propranolol) and 10 μM GTP, as indicated. [^{32}P]cAMP and [^{3}H]IP_2 were isolated and counted and the amounts present before incubation were subtracted. Values for [^{32}P]cAMP and [^{3}H]IP_2 formed from triplicate samples in incubations of control membranes are means ± S.E.M. and means ± S.E.D., respectively. Values are means from duplicate samples in incubations of pertussis toxin-treated membranes. Both the percent inhibition of adenylate cyclase and percent stimulation of phospholipase C activities by agonists added with GTP were calculated with respect to the values in incubation mixtures containing no additions; mean values are given (± S.E.Q. when triplicate incubations were performed).

Expt.	Treatment with pertussis toxin	Extent of [^{32}P]ADP-ribosylation (%)	Additions	Adenylate cyclase activity		Phospholipase C activity	
				[^{32}P]cAMP formed (nmol/10 min/ mg protein)	Inhibition (%)	[^{3}H]IP_2 formed dpm/10 min/ mg protein)	Stimulation (%)
1	None	0	None	0.271 ± 0.012	-	11,440 ± 343	-
			PAF + GTP	0.032 ± 0.002	88 ± 1	33,649 ± 276	194 ± 9
	40 µg/ml	84	None	0.127 ± 0.004	-	2,034 ± 199	
			PAF + GTP	0.099 ± 0.002	22	5,925 ± 212	191
2	None	0	None	0.139 ± 0.004	-	864 ± 85	-
			PAF + GTP	0.018 ± 0.001	87 ± 1	11,074 ± 442	1,182 ± 136
			EPI + GTP	0.036 ± 0.001	74 ± 1	1,039 ± 134	20 ± 20
	50 µg/ml	70	None	0.064 ± 0.002	-	692 ± 108	
			PAF + GTP	0.024 ± 0.002	63	7,830 ± 200	1,032
			EPI + GTP	0.058 ± 0.005	9	-	-
3	None	0	None	0.136 ± 0.003	-	4,429 ± 226	-
			PAF + GTP	0.018 ± 0.001	87 ± 1	21,000 ± 173	374 ± 25
			EPI + GTP	0.040 ± 0.001	71 ± 1	5,196 ± 226	17 ± 8
	50 µg/ml	30	None	0.080 ± 0.001	-	3,796 ± 120	-
			PAF + GTP	0.017 ± 0.001	79	17,218 ± 155	354
			EPI + GTP	0.033 ± 0.001	59	-	-

4.5.

Expt.	Treatment with the S_1 subunit of pertussis toxin	Extent of [^{32}P]ADP-ribosylation (%)	Additions	Adenylate cyclase activity		Phospholipase C activity	
				[^{32}P]cAMP formed (nmol/10 min/ mg protein	Inhibition (%)	[^{3}H]IP_2 formed (dpm/10 min/ mg protein)	Stimulation (%)
1	None	0	None	0.096 ± 0.003	-	876 ± 490	-
			PAF + GTP	0.015 ± 0.009	84 ± 9	12,649 ± 686	1,344 ± 811
			EPI + GTP	0.021 ± 0.000	78 ± 1	1,124 ± 510	28 ± 92
	10 µg/ml	91	None	0.070 ± 0.000	-	678 ± 632	-
			PAF + GTP	0.020 ± 0.002	71 ± 3	10,567 ± 784	1,459 ± 1,457
			EPI + GTP	0.025 ± 0.003	64 ± 3*	1,368 ± 719	102 ± 216
2	None	0	None	0.115 ± 0.006	-	2,742 ± 279	-
			PAF + GTP	0.021 ± 0.002	82 ± 2	17,537 ± 816	540 ± 72
			EPI + GTP	0.027 ± 0.001	77 ± 2	2,358 ± 337	-14 ± 15
	10 µg/ml	93	None	0.073 ± 0.003	-	1,947 ± 263	-
			PAF + GTP	0.031 ± 0.002	58 ± 3*	15,216 ± 447	681 ± 108
			EPI + GTP	0.048 ± 0.003	34 ± 5*	2,216 ± 242	14 ± 20
3	None	0	None	0.127 ± 0.009	-	6,087 ± 753	-
			PAF + GTP	0.032 ± 0.002	75 ± 2	15,797 ± 493	159 ± 33
			EPI + GTP	0.042 ± 0.003	67 ± 3	5,536 ± 420	-9 ± 13
	10 µg/ml	94	None	0.081 ± 0.002	-	4,915 ± 326	-
			PAF + GTP	0.048 ± 0.003	41 ± 4*	13,716 ± 400	179 ± 20
			EPI + GTP	0.068 ± 0.001	17 ± 2*	5,589 ± 389	14 ± 11

Table 4.6. Effects of treatment of rabbit platelet membranes with NEM on the actions of PAF and GTP on membrane adenylate cyclase and phospholipase C activities in suspensions containing either NaCl or KCl

Membranes from platelets labelled with [^{3}H]inositol as described in Section 2.2.9. were washed once by homogenization with hypotonic buffer and then incubated for 15 min at 25^{o}C with hypotonic buffer containing either no other additions or 50 μM NEM. The alkylation of membrane thiol residues was stopped by a 5 min incubation at 25^{o}C with hypotonic buffer containing 150 μM DTT followed by immediate centrifugation. The membranes were then resuspended for assay (Section 2.2.9.). Samples containing 0.1-0.2 mg protein were assayed for adenylate cyclase and phospholipase C activities in 10 min incubations at 25^{o}C, as described in Section 2.2.10. [α-^{32}P]ATP (2 μCi) was included in those samples from which [^{32}P]cAMP were to be isolated. Final concentrations of the additions were 2 nM and 1 μM PAF and 10 μM GTP, as indicated. [^{32}P]cAMP and [^{3}H]IP_2 were isolated and counted and the amounts present before incubation were subtracted. Values for [^{32}P]cAMP and [^{3}H]IP_2 formed in triplicate incubation mixtures are means $\pm$ S.E.M. and means $\pm$ S.E.D, respectively. Both the percent inhibition of adenylate cyclase ($\pm$ S.E.Q.) and percent stimulation of phospholipase C ($\pm$ S.E.Q.) by agonists were calculated with respect to the values in incubation mixtures containing GTP alone. Significant changes induced by NEM treatment are marked (*, $P < 0.05$; unpaired t test).

4.6.

Expt.	NEM (μM)	Salt	Additions	Adenylate cyclase activity		Phospholipase C activity	
				[^{32}P]cAMP formed (nmol/10 min/ mg protein)	Inhibition with respect to GTP control (%)	[^{3}H]IP$_2$ formed (dpm/10 min/ mg protein	Stimulation with respect to GTP control (%)
1.	None	NaCl	None	0.276 ± 0.076	-	3,827 ± 654	-
			GTP	0.210 ± 0.006	-	5,058 ± 288	-
			PAF(2 nM) + GTP	0.074 ± 0.008	65 ± 4	6,173 ± 452	22 ± 11
			PAF(1 μM) + GTP	0.012 ± 0.003	94 ± 1	20,596 ± 990	307 ± 30
	50		None	0.085 ± 0.005	-	3,918 ± 139	-
			GTP	0.130 ± 0.010	-	3,025 ± 98	-
			PAF(2 nM) + GTP	0.121 ± 0.003	7 ± 8*	3,156 ± 270	4 ± 10
			PAF(1 μM) + GTP	0.056 ± 0.005	57 ± 5*	7,779 ± 328	157 ± 14*
	None	KCl	None	0.155 ± 0.006	-	5,754 ± 123	-
			GTP	0.134 ± 0.008	-	7,877 ± 132	-
			PAF(2 nM) + GTP	0.067 ± 0.008	50 ± 7	12,281 ± 868	56 ± 11
			PAF(1 μM) + GTP	0.013 ± 0.001	90 ± 1	24,149 ± 149	207 ± 5
	50		None	0.110 ± 0.005	-	3,172 ± 303	-
			GTP	0.176 ± 0.005	-	4,566 ± 162	-
			PAF(2 nM) + GTP	0.126 ± 0.012	28 ± 7*	4,242 ± 141	-7 ± 5*
			PAF(1 μM) + GTP	0.079 ± 0.005	55 ± 3*	8,778 ± 333	92 ± 10*

4.6. cont.

Expt.	NEM (μM)	Salt	Additions	Adenylate cyclase activity		Phospholipase C activity	
				[^{32}P]cAMP formed (nmol/10 min/ mg protein)	Inhibition with respect to GTP control (%)	^{3}H]IP$_2$ formed (dpm/10 min/ mg protein	Stimulation with respect to GTP control (%)
2	None	NaCl	None	0.225 ± 0.002	-	2,220 ± 98	-
			GTP	0.234 ± 0.007	-	3,358 ± 220	-
			PAF(2 nM) + GTP	0.165 ± 0.010	29 ± 5	6,919 ± 268	106 ± 16
			PAF(1 μM) + GTP	0.017 ± 0.001	93 ± 1	41,033 ± 740	1,122 ± 83
	50		None	0.119 ± 0.001	-	896 ± 200	-
			GTP	0.178 ± 0.008	-	1,722 ± 235	-
			PAF(2 nM) + GTP	0.150 ± 0.008	16 ± 6	3,061 ± 391	78 ± 33
			PAF(1 μM) + GTP	0.081 ± 0.001	55 ± 2*	14,617 ± 348	749 ± 118
	None	KCl	None	0.083 ± 0.014	-	4,937 ± 474	-
			GTP	0.054 ± 0.025	-	9,937 ± 379	-
			PAF(2 nM) + GTP	0.046 ± 0.013	15 ± 46	32,063 ± 284	223 ± 13
			PAF(1 μM) + GTP	0.006 ± 0.002	89 ± 6	68,916 ± 747	594 ± 27
	50		None	0.091 ± 0.001	-	1,766 ± 329	-
			GTP	0.124 ± 0.013	-	2,437 ± 51	-
			PAF(2 nM) + GTP	0.109 ± 0.009	12 ± 12	5,848 ± 797	140 ± 33
			PAF(1 μM) + GTP	0.046 ± 0.009	63 ± 8	15,854 ± 184	551 ± 16

Chapter 5

Effects of PAF and epinephrine on cAMP formation in intact human platelets

5.1 *Introduction*

5.1.1. *Effects of PAF on cAMP formation in intact rabbit and human platelets*

PAF, a potent aggregating agent with both rabbit and human platelets (Cazenave *et al.*, 1979), has also been shown to be an effective inhibitor of the cAMP formation caused by PGE_1 in rabbit platelets (Haslam and Vanderwel, 1982). This was true whether or not the platelets were incubated with indomethacin and CP/CPK to prevent prostaglandin synthesis and any effects of released ADP. However, preliminary results have indicated that PAF has no effect on cAMP formation caused by PGE_1 in intact human platelets incubated with indomethacin and CP/CPK (Haslam and Vanderwel, 1982). The inability of PAF to decrease cAMP accumulation in intact human platelets is surprising since this aggregating agent is an effective inhibitor of adenylate cyclase in human platelet particulate fractions (Chapter 3). This finding parallels a similar discrepancy between the effects of vasopressin on adenylate cyclase in intact human platelets and platelet membrane preparations (Vanderwel *et al.*, 1983). One purpose of the present study was therefore to determine if the failure of PAF to decrease cAMP formation in intact human platelets could be accounted for by an identifiable difference between the extracellular or intracellular environments of the intact platelet

and the experimental conditions used for the assay of adenylate cyclase activity in platelet particulate fractions.

5.1.2. *Effects of epinephrine on human platelet activation; synergism with other aggregating agents including PAF*

A number of studies have shown that non-aggregating concentrations of epinephrine potentiate platelet activation by other aggregating agents (Ardlie *et al.*, 1966; Grant and Scrutton, 1980; Cameron and Ardlie, 1982; Lalau Keraly *et al.*, 1987; Steen *et al.*, 1988; Crouch and Lapetina, 1988), including PAF (Vargaftig *et al.*, 1982). However, the mechanism by which epinephrine activates human platelets is poorly understood (See Chapter 1). Epinephrine, at non-saturating concentrations, also increased the responsiveness of human platelets that have become desensitized to PAF (Rao and White, 1982) and thrombin (Crouch and Lapetina, 1988). Conversion of densensitized platelets to a sensitive state by epinephrine must be accounted for by a mechanism independent of cyclo-oxygenase products and released ADP, as this process was not inhibited by either aspirin or CP/CPK (Rao and White, 1982; Crouch and Lapetina, 1988). The pathway by which epinephrine potentiates the action of other aggregating agents or restores the sensitivity of refractory platelets remains to be uncovered. Crouch and Lapetina (1988) have postulated that both of these effects of epinephrine are mediated by the same mechanism. As PMA blocked the

ability of epinephrine to re-establish the thrombin-induced formation of inositol phosphates and release of internal Ca^{2+} and this effect of PMA was prevented by a PKC inhibitor, Crouch and Lapetina (1988) have also hypothesized that epinephrine reverses a PKC-induced homologous desensitization of human platelets to thrombin, possibly by modifying the phosphorylation state of α_i. It is presently known that the platelet activation caused by epinephrine is specific to α_2-adrenergic receptors in the platelet (Lasch and Jakobs, 1979) and cannot be accounted for by the ability of epinephrine to inhibit adenylate cyclase (Haslam *et al.*, 1978a,b; Clare and Scrutton, 1984). In this study, the possibility of synergism between PAF and epinephrine in inhibiting the [^{3}H]cAMP formation caused by PGE_1 was studied in order to determine if activation of human platelets by epinephrine could couple PAF receptors to adenylate cyclase in the intact human platelet.

5.1.3. *Role of Na^+ in platelet activation*

In membrane preparations from a wide variety of cells and tissues, Na^+ enhances hormone-mediated inhibition of adenylate cyclase and decreases the affinity of the receptors linked to this enzyme (Chapter 1). In intact cells, the role of Na^+ and its site of action is less clear. The resting platelet contains a low concentration of Na^+ (42 mM) and high concentration of K^+ (99 mM) (Feinberg *et al.*, 1977). Transmembrane gradients for Na^+, K^+

and Ca^{2+} exist in the platelet and are maintained by the ouabain-sensitive Na^+K^+ ATPase, a Ca^{2+}/Na^+ exchanger and the Na^+/H^+ antiport in the platelet membrane (Sandler *et al.*, 1980). Upon platelet activation, an increase in membrane permeability to Na^+ and significant Na^+ uptake was first observed with ADP (Sander *et al.*, 1980). Studies of the action of Na^+ in platelet activation have demonstrated that Na^+_o participates indirectly in secretion caused by epinephrine, ADP and low concentration of thrombin (Sweatt *et al.*, 1985). Since the amiloride analogues blocked AA release and platelet aggregation caused by platelet agonists with the same efficacy as that of Na^+/H^+ exchange, it has been postulated by Sweatt *et al.* (1985) that receptor-mediated platelet activation can be initiated by intracellular alkalinization. This was apparently due to the activation of a phosphatidylinositol-specific PLA_2, as a result of intraplatelet alkalinization due to agonist-accelerated Na^+/H^+ exchange (Sweatt *et al.*, 1986b).

In human platelets, depletion of Na^+_o had no significant effect on the inhibitory action of epinephrine on cAMP formation (Connolly and Limbird, 1983; Sweatt *et al.*, 1986b). In contrast to the above studies, Motulsky and Insel (1983) have demonstrated that Na^+_i decreases the affinity of epinephrine for its receptor in human platelets. One objective of the present study was to determine if Na^+_o and/or Na^+_i regulate stimulation and/or

inhibition of cAMP formation and to investigate if PAF receptors could couple to adenylate cyclase in intact platelets under specific concentrations of Na^+_o or Na^+_i.

5.1.4. *Manipulation of receptor-effector coupling: actions of membrane-fluidizing agents and compounds that affect the platelet cytoskeleton on adenylate cyclase activity*

Coupling of hormone receptors to adenylate cyclase requires the interaction of at least three membrane proteins, the receptor (R), G protein (G_s and/or G_i) and catalytic unit (C) (Chapter 1). Receptors are transmembrane proteins that may diffuse laterally in the lipid bilayer of the plasma membrane and activate adenylate cyclase by colliding with the G_sC components of this enzyme system (Rimon *et al*, 1978). Although some receptors, such as that for adenosine in the turkey erythrocyte, appear to be permanently coupled to adenylate cyclase, potentiation of hormone and guanine nucleotide activation of the enzyme by membrane-fluidizing agents supports a 'collision coupling' hypothesis of hormone action (Rimon *et al*, 1978; Briggs and Lefkowitz, 1980; Needham and Houslay, 1982; Levitzki, 1987a) and the concept of mobile receptors.

The fluidity of the plasma membrane is measured by fluorescence anisotropy and electron spin resonance of appropriate probes inserted into the lipid bilayer (Salesse and Garnier, 1984)

and can be altered by changing the temperature or by adding anesthetics, fatty acids, alcohols or cations (Houslay and Gordon, 1983). The most common membrane-fluidizing agents that have been used in studies of adenylate cyclase are *cis*-vaccenic acid, benzyl alcohol and ethanol (Salesse and Garnier, 1984; Houslay and Gordon, 1983). Thus, incubation of turkey erythrocytes with *cis*-vaccenic acid enhanced the rate of activation of adenylate cyclase by epinephrine (Rimon *et al.*, 1978) and restored the ability of isoproterenol to activate the enzyme in membranes that were assayed at 20°C (Briggs and Lefkowitz, 1980). Studies with benzyl alcohol have demonstrated increases in the activation of adenylate cyclase in rat liver plasma membranes (Houslay and Gordon, 1983; Gordon *et al.*, 1980). In all cases, these fluidizing agents were much more effective in potentiating adenylate cyclase stimulated by hormones, guanine nucleotides or F^- than basal enzyme activity. Although the site of action of these fluidizing agents is unclear, these studies indicate that membrane fluidity is important for effective coupling of agonists to adenylate cyclase in a number of cells. Intact human platelets were therefore incubated with *cis*-vaccenic acid or benzyl alcohol to determine if PAF receptors would couple to adenylate cyclase in membranes with enhanced fluidity.

Measurement of both the lateral and rotational diffusion of a number of membrane proteins including the EGF receptor suggests that

lateral diffusion of protein molecules is dictated by factors other than membrane fluidity (Schlessinger, 1983). Since larger diffusion rates for the lateral mobility of membrane proteins were observed in artifical lipid bilayers and membrane blebs than in intact cells, the cytoskeletal system may hinder the mobility of membrane proteins (Schlessinger, 1983). The platelet cytoskeleton consists of a membrane skeleton composed of short microfilaments just beneath the plasma membrane, followed by a layer of microtubules and then a network of cytoplasmic actin filaments (reviewed by Fox, 1987). The membrane skeleton forms a shell that maintains the shape, the lipid-protein bilayer and allows the movement of glycoproteins in the plasma membrane. Accessory proteins regulate the formation of microfilaments (profilin, gelsolin), their distribution and attachment to the plasma membrane (P235, vinculin, α-actinin, spectrin, actin binding protein) and interaction with membrane glycoproteins (P235, actin binding protein) (reviewed by Fox, 1987). For example, actin-binding protein and P235 link the membrane skeleton to the plasma membrane by binding microfilaments to glycoproteins Ib-IX (GPIb, Von Willibrand factor receptor) and IIb-IIIa (fibrinogen receptor), respectively (reviewed by Fox, 1987). Besides controlling cell shape, the cytoskeletal system is thought to play an important role in communication of information from the outside to the interior of the cell (Zor, 1983; Wu and Stracher, 1985). Electron micrographs of resting and stimulated platelets have shown that both actin and tubulin, which assemble to

form the microfilaments and microtubules, respectively, undergo major reorganization upon platelet stimulation (Geiger, 1983; Peerschke, 1984; Fox, 1987). The membrane skeleton is severed and loses its attachment to the plasma membrane in aggregated platelets upon cleavage of P235, actin binding protein and spectrin by the Ca^{2+}-activated protease (reviewed by Fox, 1987).

Accumulating biochemical, pharmacological and immunological evidence suggests that the cytoskeletal system is intimately involved in the synthesis, degradation and biological actions of cAMP (Zor, 1983). The elements of the cytoskeletal system are thought to play a role in the lateral mobility of the receptor and its coupling to G_s and C (Zor, 1983). Thus, differential detergent extraction and immunoblotting have indicated that 65% of the $\beta\gamma$ subunits in S49 lymphoma cells are associated with the microfilaments (Carlson *et al.*, 1986). In erythrocytes, α_s has also been found to be associated with the cytoskeleton (Sahyoun *et al.*, 1981 a,b). Incubation of a number of cells in the cold or with colchicine to disassemble the microtubules, enhanced hormone-stimulated adenylate cyclase but has no effect on basal enzyme activity (Zor, 1983). Agents which induce depolymerization of microtubules (cold, colchicine) and microfilaments (cytochalasins) have also been found to partially restore the sensitivity of cells to hormones after desensitization (Zor, 1983; Peerschke, 1984). Since components of the cytoskeletal system are removed from membrane preparations that

have been homogenized and washed in the cold, the failure of PAF to decrease cAMP formation caused by PGE_1 in intact human platelets could be due to constraints imposed by the cytoskeleton on the interaction of PAF receptors with the components of the adenylate cyclase system. Human platelets were therefore incubated with demecolcine or cytochalasin B to determine if disruption of the microtubules or microfilaments, respectively, would facilitate coupling of PAF receptors to adenylate cyclase.

5.1.5. *Structure-activity relationships for the effects of PAF and analogues on platelet aggregation and cAMP formation*

Previous studies with rabbit platelets have shown that the induction of platelet aggregation and inhibition of adenylate cyclase (Haslam and Vanderwel, 1982) by PAF and analogues shows high structural and stereospecificity. The activities of the compounds tested as inhibitors of adenylate cyclase (Haslam and Vanderwel, 1982) appeared to correlate with their effectiveness as inducers of platelet aggregation (Hadvary and Baumgartner, 1983). In the present study, I have directly compared the activities of PAF and analogues as inhibitors of human platelet particulate fraction adenylate cyclase with their activities as inducers of human platelet aggregation to determine whether similar receptors mediate both effects.

5.2. *Results*

5.2.1. *Failure of PAF to decrease the [^{3}H]cAMP formation caused by PGE_1 in intact human platelets*

Measurement of [^{3}H]cAMP formation was carried out using human and rabbit washed platelets that had both been prelabelled with [^{3}H]adenine and incubated with indomethacin to prevent the formation of prostaglandin endoperoxides or TxA_2 (see Table 5.1 for a representative expt.). In this study, the basal level of [^{3}H]cAMP in the absence of any activator of adenylate cyclase or inhibitor of cAMP-phosphodiesterase activity was slightly lower in rabbit than human platelets (0.021% verses 0.037% of platelet ^{3}H, mean values from 2 expts.). Incubation of washed platelets with 0.2 μM PGE_1 for 0.5 min increased the accumulation of [^{3}H]cAMP 17-fold (mean value, 2 expts.) in both rabbit and human platelets. However, further addition of 1 mM IBMX to the washed platelet suspensions, increased the accumulation of [^{3}H]cAMP over 0.5 min by another 8-fold (mean value, 2 expts.) in rabbit platelets and 1.5-fold (mean value, 2 expts.) in human platelets. Thus, it is possible that rabbit platelets may have a more active cAMP phosphodiesterase than human platelets.

When washed rabbit platelets were incubated with 0.4 μM PAF, the accumulation of [^{3}H]cAMP caused by PGE_1 was attenuated

in both the presence and absence of IBMX but PAF failed to decrease the formation of [^{3}H]cAMP caused by PGE_1 in suspensions of washed human platelets (Table 5.1.). In a previous investigation using rabbit platelets, Haslam and Vanderwel (1982) showed that ADP released from the platelet contributed to the decrease in [^{3}H]cAMP accumulation in platelets exposed to PAF. Incubation of human platelets with CP and CPK to remove released ADP, blocked the inhibition of adenylate cyclase by exogenous ADP (10 μM) (See Table 5.2) but had no effect on the inhibitory action of epinephrine. CP/CPK was included with indomethacin in studies investigating the failure of PAF to decrease [^{3}H]cAMP in the intact human platelet so that any decreases in [^{3}H]cAMP in the presence of PAF in treated platelets would not be due to the inhibition of adenylate cyclase by released ADP. Thus, in human platelets preincubated with indomethacin, CP and CPK, the inhibition of PGE_1-induced [^{3}H]cAMP formation by 400 nM PAF was -1 $\pm$ 4% (mean $\pm$ S.E.M., 3 expts.). However, 10 μM epinephrine (mixed with 10 μM propranolol to block any β-adrenergic activation of adenylate cyclase) reduced the accumulation of [^{3}H]cAMP caused by PGE_1 to a similar degree in rabbit and human platelets (Table 5.1.).

Incubation of washed rabbit and human platelet suspensions with the cAMP phosphodiesterase inhibitor, IBMX, decreased the inhibitory action of epinephrine on accumulation of [^{3}H]cAMP caused

by PGE_1 in both species and that of PAF in rabbit platelets (Table 5.1.). The latter findings are in agreement with those of Haslam and Vanderwel (1982) and support the hypothesis that PAF and epinephrine attenuate elevated levels of cAMP both by inhibiting adenylate cyclase and activating cAMP phosphodiesterase.

To exclude the possibility that the failure of PAF to decrease the accumulation of [^{3}H]cAMP in human platelets caused by PGE_1 was attributable to the platelet washing procedure, studies were carried out with heparinized PRP. In these experiments, epinephrine was a potent inhibitor of [^{3}H]cAMP formation caused by PGE_1 and PAF was without effect (Table 5.3.). Since PAF causes washed human platelets to change shape and aggregate (see Chapter 6; Fig. 6.4.), it is unlikely that the inability of PAF to inhibit adenylate cyclase in the intact human platelet is a result of loss of PAF receptors or their desensitization.

One difference between the methods for measuring adenylate cyclase activity in platelet particulate fractions and that for assaying the formation of [^{3}H]cAMP in intact platelets prelabelled with [^{3}H]adenine was the temperature at which the incubations were performed. To determine if the higher temperature used to measure [^{3}H]cAMP formation in intact platelets (37^{o}C) was responsible for the lack of effect of PAF on human platelets, [^{3}H]cAMP formation caused by PGE_1 was assayed in the presence and absence of PAF or

epinephrine at both 30°C and 37°C (Table 5.4.). Although [^{3}H]cAMP accumulation was greater at the lower temperature, PAF failed to inhibit the action of PGE_1 at either temperature, whereas epinephrine was an equally potent inhibitor under both conditions (Table 5.4.).

Although PAF was ineffective in inhibiting [^{3}H]cAMP formation in human platelets, exposure of washed platelet suspensions to maximal concentrations of PAF in the presence of a sub-optimal dose of epinephrine caused decreases in PGE_1-stimulated [^{3}H]cAMP formation that were sometimes slightly larger that when epinephrine was added alone (e.g. Table 5.7.). Since PAF and epinephrine induce human platelet aggregation in a synergistic manner when added together (Vargaftig *et al.*, 1982), a wide range of epinephrine concentrations were studied in the absence and presence of 40 nM and 400 nM PAF (Fig. 5.1.). However, no significant potentiation of the action of epinephrine by PAF was detected (one-way ANOVA, $P > 0.05$). Therefore, epinephrine does not consistently influence the coupling of PAF receptors to adenylate cyclase.

5.2.2. *Attempts to couple PAF receptors to inhibition of adenylate cyclase in intact human platelets*

Studies designed to determine optimal conditions for inhibition of adenylate cyclase by PAF in rabbit and human

particulate fractions have shown that Na^+ potentiates the inhibitory action of PAF (Chapter 3). Thus, it is possible that Na^+ regulates the ability of PAF to decrease [^{3}H]cAMP formation caused by PGE_1 in the human platelet. To investigate this possibility, it was necessary to modify both the extracellular and intracellular concentrations of platelet Na^+. The extracellular Na^+ concentration (Na^+_o) was decreased by resuspending platelets in a Tyrode's solution in which choline salts replaced Na^+ salts (Motulsky and Insel, 1983; Connolly and Limbird, 1983). The intracellular concentration of Na^+ (Na^+_i) was increased by resuspending platelets in Tyrode's solution containing a final Na^+ concentration of 150 mM and then incubating the platelet suspension for 5 min with 40 μM monensin to induce entry of Na^+ from the suspending medium into the platelet (Motulsky and Insel, 1983; Feinstein *et al.*, 1977). To prevent extrusion of Na^+, 1 μM ouabain was included in this incubation mixture to block the platelet Na^+ K^+ ATPase (Motulsky and Insel, 1983; Sandler *et al.*, 1980). Samples containing each of the four different platelet suspensions resulting from the above manipulations, were assayed for [^{3}H]cAMP formation in the absence and presence of activators and inhibitors of adenylate cyclase (Table 5.5.). Although basal [^{3}H]cAMP was not altered under any condition, [^{3}H]cAMP formation caused by PGE_1 in the absence and especially the presence of IBMX was enhanced in the platelet suspensions that were incubated with monensin and ouabain to increase Na^+_i. However, a decreased

Na^+_o had no effect on [^{3}H]cAMP formation caused by PGE_1 in the absence or presence of IBMX. Under all conditions, PAF failed to decrease [^{3}H]cAMP formation caused by PGE_1. A decrease in Na^+_o slightly diminished the inhibitory action of epinephrine on [^{3}H]cAMP formation caused by PGE_1 and IBMX. However, there was no effect on the inhibition by epinephrine of [^{3}H]cAMP formation caused by PGE_1 alone. Thus, despite the small effects of increasing Na^+_i and decreasing Na^+_o on stimulation and inhibition of adenylate cyclase by PGE_1 and epinephrine, respectively, lack of Na^+ does not appear to account for the inability of PAF to decrease [^{3}H]cAMP formation in intact human platelets.

In an attempt to promote coupling between the PAF receptors and the other components of the adenylate cyclase system, washed platelet suspensions were incubated with membrane-fluidizing agents, previously reported to enhance hormonal stimulation of adenylate cyclase. However, in this study, treatment of human platelets with benzyl alcohol and *cis*-vaccenic acid increased basal [^{3}H]cAMP levels and significantly decreased the accumulation of [^{3}H]cAMP caused by PGE_1 in the presence of IBMX (Table 5.6.). Unexpectedly, benzyl alcohol reduced the inhibition of PGE_1-stimulated [^{3}H]cAMP accumulation by epinephrine from 84% to 50%. In washed platelet suspensions that had been incubated with *cis*-vaccenic acid, the formation of [^{3}H]cAMP caused by PGE_1 in the absence or presence

of epinephrine was suppressed and no change in the % inhibition by epinephrine was observed. There was no decrease in the [^{3}H]cAMP formation caused by PGE_1 on addition of PAF with either of the above treatments.

The possibility that the microtubules or microfilaments beneath the plasma membrane interfere with the coupling of PAF receptors to adenylate cyclase in the intact platelet was examined next. Treatment of platelets with 10 μM demecolcine at 4°C for 30 min caused microtubule depolymerization and prevented repolymerization on warming, as shown by the failure of platelets to re-assume a discoid shape. However, no effects on the [^{3}H]cAMP formation caused by PGE_1 in the absence or presence of epinephrine or PAF were detected (Table 5.7.). Treatment of human platelets with different concentrations of cytochalasin B to induce depolymerization of microfilaments was also without effect (Table 5.8.).

5.2.3. *Comparison of the abilities of PAF and analogues to inhibit adenylate cyclase and induce platelet aggregation*

A preliminary study of the actions of PAF and analogues on human platelet particulate fractions and on intact human platelets (Table 5.9.) demonstrated that the ratios of activities of these compounds were similar for inhibition of adenylate cyclase and induction of platelet aggregation. However, higher agonist

concentrations were necessary for half-maximal aggregation of platelets in plasma than for half-maximal inhibition of particulate fraction adenylate cyclase. The EC_{50} value for the induction of platelet aggregation by PAF was 0.038 μM and the IC_{50} value for the inhibition of human platelet particulate fraction adenylate cyclase by PAF was 0.009 μM. Since these compounds varied widely in chemical structure and biological activity, the results are consistent with the view that the actions of PAF in inducing inhibition of platelet adenylate cyclase and platelet aggregation are mediated by a common receptor.

5.3. *Discussion*

In intact rabbit platelets and in particulate fractions from both rabbit and human platelets, PAF decreases [^{3}H]cAMP formation by inhibiting adenylate cyclase. In the rabbit platelet, the observed decreases in [^{3}H]cAMP formation are also thought to be partly due to activation of cAMP phosphodiesterase, since IMBX reduced the inhibitory action of PAF (Haslam and Vanderwel, 1982). These findings suggest that the actions of PAF on rabbit platelets are similar to those of epinephrine and ADP on rabbit and human platelets, as the latter inhibit adenylate cyclase (Marquis *et al.*, 1970; Haslam, 1973; Jakobs *et al.*, 1976; Steer and Wood, 1979; Cooper and Rodbell, 1979; Mellwig and Jakobs, 1980) and also appear to activate cAMP phosphodiesterase (Haslam and Vanderwel, 1982).

In contrast to the inhibitory action of PAF on cAMP formation in rabbit platelets and in rabbit and human platelet particulate fractions, maximal doses of PAF have no effect on the [^{3}H]cAMP formation caused by PGE_1 in washed human platelets or heparinized human PRP. Since washed platelet preparations show shape change and aggregation on exposure to PAF, it is unlikely that the lack of responsiveness of adenylate cyclase to PAF in intact platelets is due to receptor loss or damage during isolation and preparation of the platelets. The difference in incubation temperature used for measurement of [^{3}H]cAMP formation in the intact platelet and of adenylate cyclase activity in particulate fractions could not account for the discrepancy. This was not unexpected considering that epinephrine, ADP and PAF inhibit rabbit platelet adenylate cyclase under both conditions. Also, the unresponsiveness of [^{3}H]cAMP formation in human platelets to PAF cannot be accounted for by the presence PGE_1, since PAF inhibits PGE_1-stimulated adenylate cyclase activity in platelet particulate fractions (Williams *et al.*, 1984). In contrast to the ability of epinephrine to enhance platelet activation by PAF (Vargaftig *et al.*, 1982), exposure of human platelets to increasing concentrations of epinephrine had no consistent effect on the failure of PAF receptors to couple to adenylate cyclase. Even though no clear synergism between PAF and epinephrine could be detected with respect to [^{3}H]cAMP formation in human platelets, epinephrine potentiated the activation of phospholipase C by PAF in rabbit platelet particulate fractions

(Chapter 4; LaLau Keraly *et al.*, 1987; Steen *et al.*, 1988).

Since PAF inhibited adenylate cyclase in human platelet particulate fractions, attempts were made to alter the intracellular, extracellular and membrane environments and cytoskeletal structure of intact human platelets, so that [^{3}H]cAMP formation could be measured under conditions that more closely resembled those obtained in membrane preparations. In this series of experiments, the effect of PAF on [^{3}H]cAMP formation caused by PGE_1 was examined in human platelets with various concentrations of Na^+_i and Na^+_o, increased membrane fluidity and depolymerized microtubules or actin microfilaments. Since Na^+ potentiated the inhibition of adenylate cyclase by PAF in human platelet particulate fractions (Chapter 3) and this cation has been shown to be important in receptor-effector coupling in the intact human platelet (Sweatt *et al.*, 1985; 1986 a,b; Motulsky and Insel, 1983), it was surprising that neither high Na^+_i or Na^+_o permitted PAF to suppress [^{3}H]cAMP formation in the human platelet. The results suggest that Na^+ does not affect the coupling of the PAF receptor to adenylate cyclase in the intact platelet but acts only to enhance the effects observed after coupling has been established. However, the increase in PGE_1-stimulated [^{3}H]cAMP formation with high Na_i is consistent with the results obtained using membrane preparations (W. Murphy, unpublished).

Enhancement of the membrane fluidity with *cis*-vaccenic acid or benzyl alcohol or disruption of the platelet cytoskeleton with demecolcine or cytochalasin B had no effect on the failure of PAF to decrease [^{3}H]cAMP formation in human platelets treated with these agents. Although these compounds had been reported to enhance hormone-stimulated adenylate cyclase activity (Rimon *et al.*, 1978; Houslay and Gordon, 1983; Zor, 1983), the viscosity of the membrane and the platelet cytoskeleton do not appear to prevent coupling of PAF receptors to adenylate cyclase in the human platelet. Brass *et al.* (1988) recently demonstrated that PAF and AVP fail to decrease the cAMP formation caused by PGI_2 in saponin-permeabilized human platelets under conditions in which epinephrine was an effective inhibitor of adenylate cyclase. Thus, it is unlikely that a low molecular mass inhibitor is responsible for this phenomenon.

Another possible explanation for the failure of PAF to suppress the [^{3}H]cAMP formation caused by PGE_1 in human platelets that can respond to PAF with shape change, aggregation and secretion (Fouque and Vargaftig, 1984) could be that the latter actions of PAF are mediated by distinct receptors that alone are coupled to effector molecules (presumably phospholipase C) in the intact platelet. Although PAF receptor subtypes have not been demonstrated in platelets, the effects of PAF antagonists on [^{3}H]PAF binding and on PAF-stimulated GTPase and aggregation have suggested that the PAF

receptors on human platelets are distinct from those on human leukocytes (Hwang, 1988). A preliminary investigation of the structure-activity relationships for the effects of PAF and analogues on adenylate cyclase activity in human platelet particulate fractions and on the induction of human platelet aggregation demonstrated similar activity ratios for both effects, suggesting that these actions of PAF could be mediated by similar if not identical receptors. In support of this hypothesis are findings of a single class of high affinity PAF binding sites on intact human platelets (Valone *et al.*, 1982; Kloprogge and Akkerman, 1984) and on human platelet membranes (Hwang *et al.*, 1983). Since higher EC_{50} values for platelet aggregation than IC_{50} values for the inhibition of adenylate cyclase were found in the present study, it is possible that more receptors must be occupied by PAF for platelet activation than for inhibition of adenylate cyclase. Although, this interpretation is consistent with the larger doses of PAF required for half-maximal activation of phospholipase C than for half-maximal inhibition of adenylate cyclase as in rabbit platelet particulate fractions (Chapter 4), it is possible that the results reflect non-specific binding of PAF to plasma proteins in the aggregation assay. Since rabbit platelets contain a larger number of high-affinity [^{3}H]PAF binding sites than human platelets (Hwang *et al.*, 1983; Inarrea *et al.*, 1984), the difference in the ability of PAF to suppress [^{3}H]cAMP formation caused by PGE_1 in these two species could also partly be a reflection of receptor

number. Although the reported K_D values for these receptors (1.36 nM and 4.5 nM in rabbit and human platelets, respectively) (Hwang *et al.*, 1983) are different, GTP and Na^+ have been shown to decrease receptor affinity in rabbit platelet membranes (Hwang *et al.*, 1986). However, if a single receptor type mediates both actions of PAF, it is necessary to explain why the effect (inhibition of adenylate cyclase) that requires the lower PAF concentration, at least in rabbit platelet membranes, is suppressed in intact human platelets. The coupling of the same receptor type to different effectors could be regulated by the G proteins with which they interact and this could be determined by soluble protein factors lost during the isolation of membranes. In this connection, it is of interest that Hwang (1988) recently demonstrated that the G proteins coupled to the distinct PAF receptor subtypes on human platelets and leukocytes exhibit different sensitivities to cholera toxin and pertussis toxin.

The inability of an agonist to decrease cAMP formation in intact human platelets even though it is an inhibitor of adenylate cyclase in membrane preparations has been observed previously with AVP (Vanderwel *et al.*, 1983), prostaglandin endoperoxide analogues (Best *et al.*, 1979; Avdonin *et al.*, 1985) and low concentrations of thrombin (Aktories and Jakobs, 1984; McGowen and Detwiler, 1986). Similar structure-activity relationships of AVP and analogues acting as inhibitors of adenylate cyclase and as inducers

of platelet aggregation and an identical pA_2 value for inhibition of these two effects by a AVP antagonist suggested that these actions of AVP are also mediated by a single receptor type (Vanderwel *et al.*, 1983). It now appears that most agonists that directly activate platelet phospholipase C have little or no effect on cAMP formation in intact human platelets, whereas compounds which do not activate this enzyme are effective inhibitors of adenylate cyclase in the human platelet (Haslam *et al.*, 1985). Agonists which directly stimulate phospholipase C include thrombin, PAF, AVP and stable prostaglandin endoperoxides (Lapetina, 1983; MacIntyre *et al.*, 1985a). Other agonists, such as ADP (MacIntyre *et al.*, 1985; Fisher *et al.*, 1985) and epinephrine (MacIntyre *et al.*, 1985; Siess *et al.*, 1984) which have little or no direct action on phospholipase C, can both decrease cAMP formation in intact human platelets (Haslam, 1973) and inhibit adenylate cyclase activities in membrane preparations (Cooper and Rodbell, 1979: Jakobs *et al.*, 1978).

5.4. *Summary*

5.4.1. PAF did not decrease the cAMP formation caused by addition of PGE_1 in the absence or presence of IBMX in intact human platelets. This contrasts with the inhibitory action of epinephrine on cAMP formation under the same experimental conditions, the ability of PAF to attenuate cAMP formation in intact rabbit platelets and the inhibition of adenylate cyclase activity by PAF in particulate fractions prepared from both human and rabbit platelets.

5.4.2. The anomalous failure of PAF to decrease cAMP formation in intact human platelets was not due to the platelet washing procedure used, the incubation temperature or a loss of responsiveness of the platelets to PAF.

5.4.3. It was not possible to re-couple PAF receptors to adenylate cyclase in the human platelet by addition of a low epinephrine concentration, by altering the intracellular or extracellular concentration of Na^+, by increasing the membrane fluidity or by depolymerizing platelet microtubules or microfilaments.

5.4.4. The activity ratios of PAF analogues as inhibitors of adenylate cyclase and as inducters of platelet aggregation were consistent with the view that a single PAF receptor type mediates both effects.

Fig. 5.1. Effect of PAF on the dose-response curve for inhibition of PGE_1-induced [^{3}H]cAMP formation by different concentrations of epinephrine

Washed human platelets labelled with [^{3}H]adenine were prepared as described in Section 2.2.3. and [^{3}H]cAMP formation was assayed as in Section 2.2.14. The platelet suspensions were preincubated with indomethacin, CP and CPK prior to the addition of any activators and/or inhibitors of adenylate cyclase. The final concentrations of additions were 10 μM indomethacin, 2 mM CP, 100 units of CPK/ml, 0.2 μM PGE_1. All concentrations of epinephrine were added with 10 μM propranolol. Samples contained no PAF (●), 40 nM PAF (▲) or 400 nM PAF (◆). Values given for [^{3}H]cAMP formation are means ± S.E.M. from triplicate determinations.

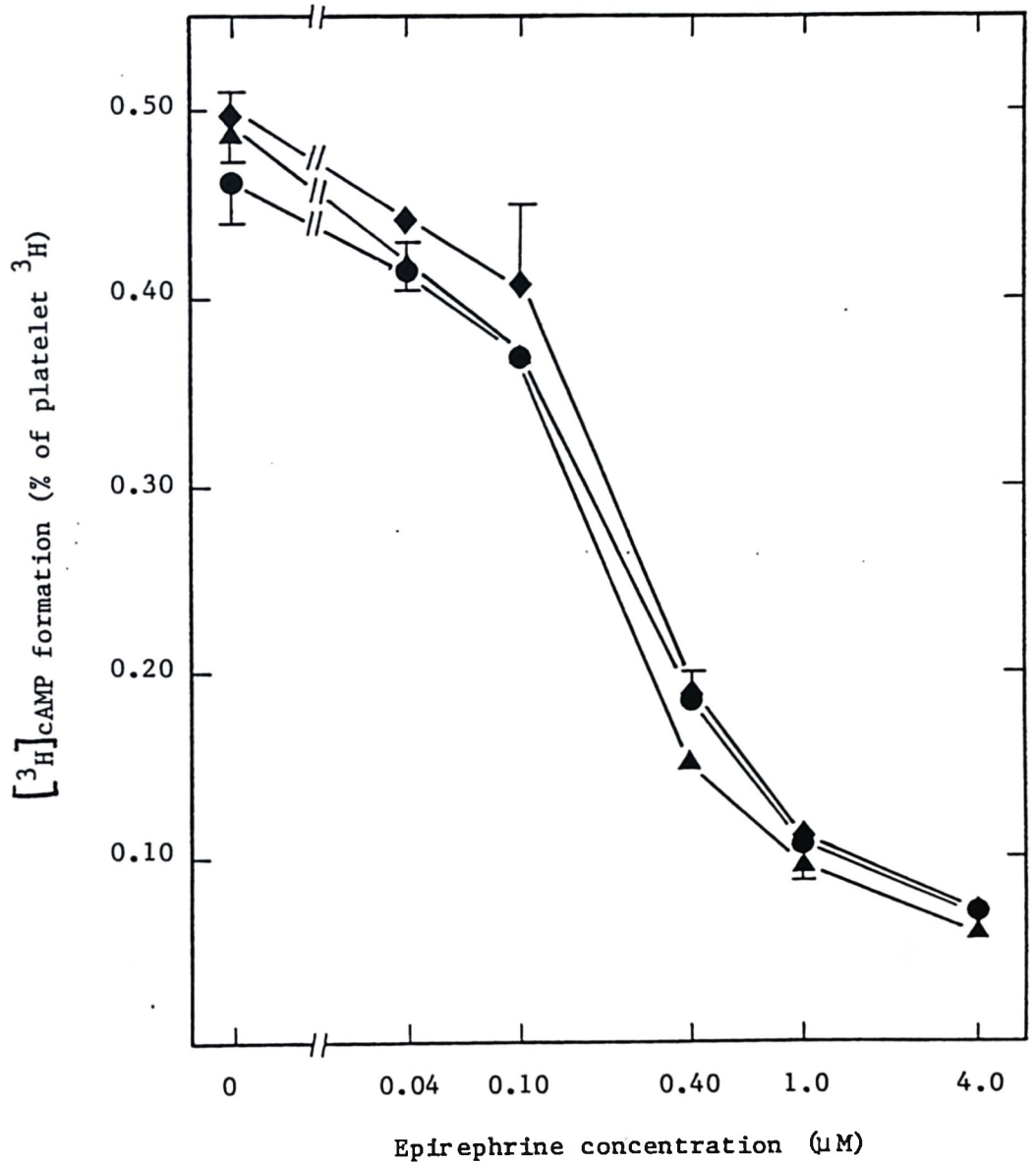

0.50
0.40
0.30
0.20
0.10
[3H]cAMP formation (% of platelet 3H)
0
0.04
0.10
0.40
1.0
4.0
Epirephrine concentration (μM)

Table 5.1. Effects of PAF and of epinephrine on the [^{3}H]cAMP formation caused by PGE_1 in washed rabbit and human platelets

The accumulation of [^{3}H]cAMP in rabbit and human washed platelets was assayed as described in Section 2.2.14.. The platelet suspensions were preincubated with indomethacin in the absence of CP/CPK for 5 min prior to the addition of any activators or inhibitors of adenylate cyclase. The final concentrations of these additions were 10 μM indomethacin, 0.2 μM PGE_1, 0.4 μM PAF and 10 μM epinephrine (with 10 μM propranolol). [^{3}H]cAMP accumulation was measured in the absence and presence of 1 mM IBMX, as indicated. Values are means ± S.E.M. from triplicate determinations. The basal levels of [^{3}H]cAMP were subtracted in calculation of the percent inhibition by PAF or epinephrine of the accumulation of [^{3}H]cAMP caused by PGE_1 in the absence or presence of IBMX (mean values ± S.E.Q. are given).

Species	Additions	[^{3}H]cAMP (% of platelet ^{3}H)	Inhibition of [^{3}H]cAMP formation (%)
Rabbit	None	0.026 ± 0.001	–
	PGE_1	0.485 ± 0.035	–
	PGE_1 + PAF	0.087 ± 0.006	87 ± 2
	PGE_1 + EPI	0.048 ± 0.003	95 ± 1
	PGE_1 + IBMX	1.776 ± 0.063	–
	PGE_1 + IBMX + PAF	0.753 ± 0.020	58 ± 2
	PGE_1 + IBMX + EPI	0.509 ± 0.007	72 ± 1
Human	None	0.036 ± 0.002	–
	PGE_1	0.540 ± 0.026	–
	PGE_1 + PAF	0.509 ± 0.056	6 ± 12
	PGE_1 + EPI	0.054 ± 0.001	94 ± 1
	PGE_1 + IBMX	0.803 ± 0.035	–
	PGE_1 + IBMX + PAF	0.740 ± 0.025	8 ± 5
	PGE_1 + IBMX + EPI	0.198 ± 0.041	79 ± 5

Table 5.2. Effects of ADP and of epinephrine on [^{3}H]cAMP formation caused by PGE_1 and IBMX in human platelets incubated with and without CP and/or CPK

The accumulation of [^{3}H]cAMP in washed human platelets was assayed as described in Section 2.2.14. The platelet suspensions were preincubated with indomethacin in the absence or presence of CP and/or CPK for 5 min prior to the addition of any activators or inhibitors of adenylate cyclase. The final concentrations of additions were 10 μM indomethacin, 2 mM CP, 100 units of CPK/ml, 0.2 μM PGE_1, 10μM ADP and 5 μM epinephrine (with 10 μM propranolol). The accumulation of [^{3}H]cAMP was measured in the absence and presence of 1 mM IBMX, as indicated. Values are means ± S.E.M. from triplicate determinations. The basal levels of [^{3}H]cAMP were subtracted for calculation of the percent inhibition by PAF or epinephrine of [^{3}H]cAMP formation caused by PGE_1 and IBMX (mean values ± S.E.Q. are given).

Incubation Conditions	Additions	[^{3}H]cAMP (% of platelet ^{3}H)	Inhibition of [^{3}H]cAMP formation (%)
- CP/CPK	None	0.033 ± 0.002	–
	PGE_1 + IBMX	1.302 ± 0.022	–
	PGE_1 + IBMX + ADP	0.336 ± 0.011	76 ± 1
	PGE_1 + IBMX + EPI	0.232 ± 0.011	84 ± 1
+ CP only	None	0.035 ± 0.001	–
	PGE_1 + IBMX	1.369 ± 0.027	–
	PGE_1 + IBMX + ADP	0.339 ± 0.009	77 ± 1
	PGE_1 + IBMX + EPI	0.228 ± 0.008	86 ± 1
+ CPK only	None	0.038 ± 0.003	–
	PGE_1 + IBMX	1.366 ± 0.057	–
	PGE_1 + IBMX + ADP	0.339 ± 0.009	77 ± 3
	PGE_1 + IMBX + EPI	0.227 ± 0.007	86 ± 1
+ CP/CPK	None	0.037 ± 0.001	–
	PGE_1 + IBMX	1.369 ± 0.013	–
	PGE_1 + IBMX + ADP	1.197 ± 0.018	13 ± 2
	PGE_1 + IBMX + EPI	0.226 ± 0.002	86 ± 1

Table 5.3. Comparison of the actions of PAF and of epinephrine on the [^{3}H]cAMP formation in human platelets suspended in heparinized plasma and Tyrode's albumin solution

Heparinized PRP and washed human platelets each labelled with [^{3}H]adenine were prepared (Section 2.2.3) and the accumulation of [^{3}H]cAMP in these platelet preparations was assayed in the presence of 10 μM indomethacin, 2 mM CP and 100 units of CPK/ml as described in Section 2.2.14. The heparinized PRP and washed human platelets were from different donors. Heparinized PRP was incubated with 10 μM epinephrine and washed human platelets with 5 μM epinephrine (both with 10 μM propranolol). The final concentrations of the other compounds added were 0.2 μM PGE_1, 0.4 μM PAF and 1 mM IBMX. Values are means ± S.E.M. from triplicate determinations. The basal levels of [^{3}H]cAMP were subtracted for calculation of the percent inhibition by PAF and epinephrine of the accumulation of [^{3}H]cAMP in the presence of either no additions, IBMX or PGE_1 (mean values ± S.E.Q. are given).

Platelet Preparation	Additions	[^{3}H]cAMP (% of platelet ^{3}H)	Inhibition of [^{3}H]cAMP formation (%)
Heparinized PRP	None	0.026 ± 0.001	–
	PGE_1	0.176 ± 0.020	–
	PGE_1 + PAF	0.198 ± 0.007	-15 ± 16
	PGE_1 + EPI	0.024 ± 0.001	101 ± 1
	PGE_1 + IBMX	0.774 ± 0.013	–
	PGE_1 + IBMX + PAF	0.779 ± 0.009	-1 ± 2
	PGE_1 + IBMX + EPI	0.126 ± 0.003	87 ± 1
Washed platelets	None	0.044 ± 0.001	–
	PAF	0.035 ± 0.003	20 ± 7
	PGE_1	0.667 ± 0.028	–
	PGE_1 + PAF	0.672 ± 0.006	-1 ± 5
	PGE_1 + EPI	0.084 ± 0.005	94 ± 1
	IBMX	0.078 ± 0.002	–
	IBMX + PAF	0.074 ± 0.005	12 ± 16
	PGE_1 + IBMX	1.761 ± 0.086	–
	PGE_1 + IBMX + PAF	1.500 ± 0.029	15 ± 5
	PGE_1 + IBMX + EPI	0.372 ± 0.018	81 ± 1

Table 5.4. Effect of incubation temperature on the actions of PAF and epinephrine on the [^{3}H]cAMP formation caused by PGE_1

Washed human platelets labelled with [^{3}H]adenine were prepared as described in Section 2.2.3. [^{3}H]cAMP formation was measured at both 37°C and 30°C using the same platelet preparation incubated with the final concentrations of 10 μM indomethacin, 2 mM CP and 100 units of CPK/ml, as described in Section 2.2.14. The concentrations of additions were 0.2 μM PGE_1, 0.4 μM PAF and 0.4 μM epinephrine (with 10 μM propranolol). The values given for [^{3}H]cAMP formation are means ± S.E.M. from triplicate determinations. The basal levels of [^{3}H]cAMP were subtracted for calculation of the percent inhibition by PAF and epinephrine of [^{3}H]cAMP formation caused by PGE_1 (mean values ± S.E.Q. are given).

Incubation temperature	Additions	[^{3}H]cAMP (% of platelet ^{3}H)	Inhibition of [^{3}H]cAMP formation (%)
37°C	None	0.023 ± 0.001	–
	PGE_1	0.463 ± 0.026	–
	PGE_1 + PAF	0.500 ± 0.011	-8 ± 7
	PGE_1 + EPI	0.186 ± 0.003	63 ± 2
	PGE_1 + PAF + EPI	0.189 ± 0.011	62 ± 3
30°C	None	0.028 ± 0.001	–
	PGE_1	0.709 ± 0.020	–
	PGE_1 + PAF	0.734 ± 0.026	-4 ± 5
	PGE_1 + EPI	0.311 ± 0.008	58 ± 2
	PGE_1 + PAF + EPI	0.277 ± 0.006	63 ± 1

Table 5.5. Effects of changes in the intracellular and extracellular Na^+ concentration on the formation of [^{3}H]cAMP caused by PGE_1 in the absence and presence of PAF or epinephrine

Washed human platelets labelled with [^{3}H]adenine were incubated in the presence of the indicated additions and assayed for the formation of [^{3}H]cAMP as described in Sections 2.2.14. To increase the intracellular concentration of Na^+, 40 μl samples of concentrated platelet suspension (5 x 10^9 platelets/ml) were mixed with 20 μl of 0.154 M NaCl containing 40 μM indomethacin and incubated for 5 min with 20 μl of 0.154 M NaCl containing 160 μM monensin and 4 μM ouabain. Similar incubations were carried out without monensin and ouabain when platelets with the usual low intracellular Na^+ were required. Extracellular and intracellular Na^+ concentrations (Na^+_o and Na^+_i, respectively) were then varied as follows: (1) high Na^+_o, low Na^+_i was achieved by diluting control platelet suspension (80 μl) into a large volume (900 μl) of Tyrode's solution containing Na^+ (at the same time as the addition of 20 μl of vehicle containing any activators and/or inhibitors of adenylate cyclase); (2) low Na^+_o, low Na^+_i was achieved by diluting platelet suspension into a large volume of Tyrode's solution containing choline instead of Na^+ salts (NaCl and $NaHCO_3$ in the suspending Tyrode's solution were replaced by choline chloride and choline bicarbonate and the solution was buffered with Hepes adjusted to pH 7.4 with Tris base instead of NaOH) (final Na^+_o, ~10-20 mM); (3) high Na^+_o, high Na^+_i was achieved by diluting platelet suspension pretreated with monensin and ouabain into a large volume of Tyrode's solution containing Na^+ (final Na^+_o, ~150 mM); (4) low Na^+_o, high Na^+_i was achieved by diluting platelet suspension pretreated with monensin and ouabain into a large volume of Tyrode's solution containing choline salts. The final concentrations of compounds used in the experiment were 0.2 μM PGE_1, 0.4 μM PAF, 10 μM epinephrine (with 10 μM propranolol) and 1 mM IBMX. The values given for [^{3}H]cAMP formation are means $\pm$ S.E.M. from triplicate determinations. The basal levels of [^{3}H]cAMP were subtracted for calculation of the percent inhibition by PAF or epinephrine of the accumulation of [^{3}H]cAMP caused by PGE_1 (mean values $\pm$ S.E.Q. are given).

5.5.

Conditions	Additions	[^{3}H]cAMP (% of platelet ^{3}H)	Inhibition of [^{3}H]cAMP formation (%)
High Na^+_o Low Na^+_i	None	0.037 ± 0.003	–
	PGE_1	0.686 ± 0.020	–
	PGE_1 + PAF	0.706 ± 0.110	-3 ± 17
	PGE_1 + EPI	0.094 ± 0.030	91 ± 5
	PGE_1 + IBMX	1.096 ± 0.026	–
	PGE_1 + IBMX + PAF	1.134 ± 0.007	-4 ± 3
	PGE_1 + IBMX + EPI	0.270 ± 0.023	78 ± 2
Low Na^+_o Low Na^+_i	None	0.041 ± 0.003	–
	PGE_1	0.722 ± 0.018	–
	PGE_1 + PAF	0.772 ± 0.028	-7 ± 5
	PGE_1 + EPI	0.106 ± 0.004	90 ± 1
	PGE_1 + IBMX	0.912 ± 0.109	–
	$PGE^{\pm}$ + IBMX + PAF	1.090 ± 0.009	-20 ± 15
	PGE_1 + IBMX + EPI	0.331 ± 0.024	67 ± 5
High Na^+_o High Na^+_i	None	0.037 ± 0.001	–
	PGE_1	0.860 ± 0.096	–
	PGE_1 + PAF	1.223 ± 0.054	-44 ± 18
	PGE_1 + EPI	0.155 ± 0.004	86 ± 2
	PGE_1 + IBMX	1.571 ± 0.037	–
	PGE_1 + IBMX + PAF	1.574 ± 0.011	0 ± 3
	PGE_1 + IBMX + EPI	0.440 ± 0.014	74 ± 1
Low Na^+_o High Na^+_i	None	0.037 ± 0.003	–
	PGE_1	1.005 ± 0.050	–
	PGE_1 + PAF	0.993 ± 0.048	1 ± 7
	PGE_1 + EPI	0.259 ± 0.019	77 ± 2
	PGE_1 + IBMX	1.490 ± 0.064	–
	PGE_1 + IBMX + PAF	1.438 ± 0.047	4 ± 5
	PGE_1 + IBMX + EPI	0.529 ± 0.016	66 ± 2

Table 5.6. Effects of membrane-fluidizing agents on the stimulation and inhibition of [^{3}H]cAMP formation in washed human platelets

Human platelets were washed, labelled with [^{3}H]adenine and assayed for [^{3}H]cAMP, as described in Sections 2.2.3. and 2.2.14.. Platelet suspensions containing indomethacin, CP and CPK were incubated with the membrane-fluidizing agents, benzyl alcohol or *cis*-vaccenic acid, for 10 min prior to the addition IBMX and of any activators and/or inhibitors of adenylate cyclase. IBMX was present in all incubations including the control. Benzyl alcohol was dissolved in the platelet resuspension medium and *cis*-vaccenic acid in 95% ethanol (final concentration 1%). The control platelet suspensions for these two treatments were incubated with the same volumes of these solvents. The final concentrations of additions were 10 μM indomethacin, 2 mM CP and 100 units of CPK/ml, 0.2 μM PGE_1, 0.1 μM PAF, 10 μM epinephrine (with 10 μM propranolol) and 1 mM IBMX. The values given for [^{3}H]cAMP formation are the means $\pm$ S.E.M. from triplicate determinations. The basal level of [^{3}H]cAMP was subtracted for calculation of the percent inhibition by PAF or epinephrine of the accumulation of [^{3}H]cAMP caused by PGE_1 (mean values $\pm$ S.E.Q. are given).

Treatment of platelet suspension	Additions	[^{3}H]cAMP (% of platelet ^{3}H)	Inhibition of [^{3}H]cAMP formation (%)
Control	None	0.055 ± 0.001	–
	PGE_1	1.600 ± 0.069	–
	PGE_1 + PAF	1.498 ± 0.030	7 ± 5
	PGE_1 + EPI	0.294 ± 0.003	84 ± 1
Benzyl Alcohol (4 mM)	None	0.059 ± 0.004	–
	PGE_1	1.714 ± 0.061	–
	PGE_1 + PAF	1.607 ± 0.035	6 ± 4
	PGE_1 + EPI	0.428 ± 0.002	78 ± 1
Benzyl Alcohol (40 mM)	None	0.090 ± 0.003	–
	PGE_1	0.870 ± 0.049	–
	PGE_1 + PAF	0.851 ± 0.061	2 ± 10
	PGE_1 + EPI	0.481 ± 0.005	50 ± 3
Control	None	0.053 ± 0.003	–
	PGE_1	1.541 ± 0.025	–
	PGE_1 + PAF	1.491 ± 0.013	3 ± 2
	PGE_1 + EPI	0.375 ± 0.005	76 ± 1
Cis-vaccenic acid (0.5 mM)	None	0.066 ± 0.001	–
	PGE_1	1.091 ± 0.000	–
	PGE_1 + PAF	1.129 ± 0.156	-4 ± 15
	PGE_1 + EPI	0.236 ± 0.003	83 ± 1
Cis-vaccenic acid (1.0 mM)	None	0.078 ± 0.007	–
	PGE_1	0.331 ± 0.061	–
	PGE_1 + PAF	0.296 ± 0.001	14 ± 21
	PGE_1 + EPI	0.145 ± 0.043	73 ± 18

Table 5.7. Effect of demecolcine on the [^{3}H]cAMP formation caused by PGE_1 in the absence and presence of PAF and/or epinephrine

Platelets were prepared and assayed for [^{3}H]cAMP as described in Sections 2.2.3. and 2.2.14. Control and treated platelet suspensions containing indomethacin, CP and CPK were incubated for 30 min at 4°C in the absence or presence of 10 µM demecolcine, respectively, followed by 30 min at 37°C prior to addition of the indicated compounds and measurement of [^{3}H]cAMP. The final concentrations of additions were 10 µM indomethacin, 2 mM CP and 100 units of CPK/ml, 0.2 µM PGE_1 and 0.4 µM PAF. The values given for [^{3}H]cAMP formation are means ± S.E.M. from triplicate determinations. The basal levels of [^{3}H]cAMP were subtracted for calculation of the percent inhibition of the accumulation of [^{3}H]cAMP caused by PGE_1 (mean values ± S.E.Q. are given).

Treatment of platelet suspension	Additions	[^{3}H]cAMP (% of platelet ^{3}H)	Inhibition of [^{3}H]cAMP formation (%)
None	None	0.046 ± 0.001	–
	PGE_1	0.795 ± 0.003	–
	PGE_1 + PAF	0.797 ± 0.020	0 ± 0
	PGE_1 + 10 µM EPI	0.092 ± 0.002	94 ± 1
	PGE_1 + 0.2 µM EPI	0.601 ± 0.007	26 ± 1
	PGE_1 + 0.2 µM EPI + PAF	0.496 ± 0.006	40 ± 1
Demecolcine	None	0.048 ± 0.001	–
	PGE_1	0.822 ± 0.021	–
	PGE_1 + PAF	0.837 ± 0.043	-2 ± 6
	PGE_1 + 10 µM EPI	0.104 ± 0.012	93 ± 1
	PGE_1 + 0.2 µM EPI	0.639 ± 0.039	24 ± 5
	PGE_1 + 0.2 µM EPI + PAF	0.530 ± 0.017	38 ± 3

Table 5.8. Effect of cytochalasin B on the [^{3}H]cAMP formation caused by PGE_1 in the absence and presence of PAF or epinephrine

Platelets were prepared and assayed for [^{3}H]cAMP as described in Sections 2.2.3. and 2.2.14. Control and treated platelet suspensions containing indomethacin, CP and CPK were incubated for 10 min at 37° with DMSO (0.2%) or 2, 10 or 50 μg of cytochalasin B/ml, respectively, prior to the addition of the indicated agonists and measurement of [^{3}H]cAMP. The final concentrations of additions were 10 μM indomethacin, 2 mM CP and 100 units of CPK/ml, 0.2 μM PGE_1, 0.4 μM PAF and 10 μM epinephrine (with 30 μM propranolol). The values given for [^{3}H]cAMP formation are means ± S.E.M. from triplicate determinations. The basal levels of [^{3}H]cAMP were subtracted for calculation of the percent inhibition by PAF or epinephrine of [^{3}H]cAMP accumulation caused by PGE_1 (mean values ± S.E.Q. are given).

Treatment of platelet suspensions	Additions	[^{3}H]cAMP (% of platelet ^{3}H)	Inhibition of [^{3}H]cAMP formation (%)
DMSO	None	0.036 ± 0.001	-
	PGE_1	0.737 ± 0.007	-
	PGE_1 + PAF	0.790 ± 0.021	-8 ± 3
	PGE_1 ± EPI	0.150 ± 0.009	84 ± 1
Cytochalasin B (2 μg/ml)	None	0.039 ± 0.001	-
	PGE_1	0.760 ± 0.002	-
	PGE_1 + PAF	0.810 ± 0.035	-7 ± 5
	PGE_1 + EPI	0.171 ± 0.006	82 ± 1
Cytochalasin B (10 μg/ml)	None	0.036 ± 0.002	-
	PGE_1	0.724 ± 0.023	-
	PGE_1 + PAF	0.817 ± 0.009	-13 ± 4
	PGE_1 + EPI	0.174 ± 0.005	80 ± 1
Cytochalasin B (50 μg/ml)	None	0.036 ± 0.001	-
	PGE_1	0.780 ± 0.027	-
	PGE_1 + PAF	0.884 ± 0.014	-14 ± 5
	PGE_1 + EPI	0.188 ± 0.002	80 ± 1

Table 5.9. Comparison of the IC_{50} values for inhibition of human platelet adenylate cyclase by PAF and analogues with the corresponding EC_{50} values for induction of platelet aggregation

Platelet particulate fractions used for assay of adenylate cyclase were prepared and assayed in the presence of 10 μM GTP and 150 mM NaCl, as indicated in Sections 2.2.7. and 2.2.8. Under these conditions, the control enzyme activity was 0.46 nmol/10 min per mg protein and 1 μM PAF caused a maximal inhibition of 60%. Concentrations of PAF causing half-maximal inhibition (30%) were determined (IC_{50}) for PAF and analogues. Heparinized PRP was prepared as in Section 2.2.1. and incubated for 10 min with 10 μM indomethacin prior to the addition of PAF and analogues. Platelet aggregation was measured turbidometrically. Concentrations of PAF and analogues causing a half-maximal rate of increase in light transmission were determined (EC_{50} values). The abbreviations G-3-PC and G-1-PC stand for glycero-3-phosphocholine and glycero-1-phosphocholine, respectively.

Compound	Inhibition of adenylate cyclase		Induction of aggregation	
	IC_{50} (μM)	Ratio	EC_{50} (μM)	Ratio
1-Octadecyl-2-acetyl-G-3-PC(PAF)	0.009	–	0.038	–
1-Octadecyl-2-propionyl-G-3-PC	0.009	1.0	0.054	1.4
1-Acetyl-2-octadecyl-G-3-PC	0.37	41	1.16	31
3-Octadecyl-2-acetyl-G-1-PC	1.4	156	2.78	73
1-Octadecyl-2-lyso-G-3-PC	>10	>1100	>10	>263

Chapter 6

Effects of activation of PKC on the agonist-induced stimulation and inhibition of cAMP formation in intact human platelets

6.1. *Introduction*

6.1.1. *Effects of activation of PKC by PMA on the stimulation and inhibition of adenylate cyclase by agonists*

Activation of PKC not only promotes cell responses but also regulates signal transduction (Drummond and MacIntyre, 1985; Nishizuka, 1986). PMA has been shown to inhibit (Garte and Belman, 1980; Heyworth *et al.*, 1984; Kelleher *et al.*, 1984; Kassis *et al.*, 1985; Quilliam *et al.*, 1985) or enhance (Quilliam *et al.*, 1985; Sugden *et al.*, 1985; Bell *et al.*, 1985; Jakobs *et al.*, 1985; Yoshimasa *et al.*, 1987; Rozengurt *et al.*, 1987; Langlois *et al.*, 1987) hormonal stimulation of adenylate cyclase, the precise effect depending on the cell and agonist studied, as well as the experimental conditions. Johnson *et al.* (1986) discovered that the period of incubation and the the dose of PMA, as well as the concentration of stimulatory agonist, were critical to the effect of PMA on adenylate cyclase.

Cross-talk between the phosphoinositide and adenylate cyclase systems was first indicated by the potentiation of β-adrenergic receptor-stimulated cAMP production by occupation of α_1-adrenergic receptors in pinealocytes (Sugden *et al.*, 1985) and by the synergistic effects of Ca^{2+}-mobilizing and cAMP

generating agonists on adenylate cyclase activity in brain (Hollingsworth and Daly, 1985). The stimulatory action of occupation of Ca^{2+}-mobilizing receptors on the increases in cAMP caused by β-adrenergic agonists has been mimicked by PMA in a number of cells, suggesting that PKC can stimulate the adenylate cyclase system (Sugden *et al.*, 1985; Sibley *et al.*, 1986; Bell *et al.*, 1985).

In contrast, to these stimulatory actions of PMA and Ca^{2+}-mobilizing agonists, decreases in cAMP formation were observed in PMA-treated platelets (Zucker *et al.*, 1974; Ashby *et al.*, 1985). However, the inhibitory action of PMA was attributed to the release of ADP (Ashby *et al.*, 1985). Also, PMA enhanced the activation of adenylate cyclase by PGE_1 and diminished the inhibition of the enzyme by epinephrine (Jakobs *et al.*, 1985). Both effects were attributed to the impairment of signal transduction by the phosphorylation of the α-subunit of G_i by PKC (Katada *et al.*, 1985). Jakobs *et al.* (1985) suggested that this action of PKC may explain why some agonists that activate phospholipase C do not inhibit adenylate cyclase in intact platelets (e.g. AVP, as reported by Vanderwel *et al.*, 1983).

6.1.2. *Objectives of the study*

The purpose of this study was therefore to determine if

activation of PKC by Ca^{2+}-mobilizing agonists was responsible for the failure of AVP or PAF to decrease cAMP formation caused by PGE_1 in the intact human platelet. This hypothesis was tested by studying the relationship between the activation of PKC by PMA, diC_8 and platelet agonists and the effects of these compounds on cAMP formation in intact human platelets. A novel double-labelling assay in which human platelets were prelabelled with [^{3}H]adenine and [^{32}P]P_i, was developed so that both [^{3}H]cAMP formation and incorporation of ^{32}P into platelet PKC substrates could be determined in the same sample.

6.2. *Results*

6.2.1. *Effects of PMA and analogues on the agonist-induced stimulation of [^{3}H]cAMP formation*

Experiments were carried out in the presence of indomethacin to prevent the formation of prostaglandin endoperoxides or TxA_2 and of CP and CPK to remove any ADP released from the platelets. These precautions were necessary to ensure that changes in platelet [^{3}H]cAMP formation were due solely to direct effects of the added compounds. Under these conditions, [^{3}H]cAMP accumulation in the presence of PGE_1 and IBMX in intact human platelets prelabelled with [^{3}H]adenine was linear for about 30 s (Fig. 6.1.) and was therefore assumed to reflect the endogenous adenylate cyclase

activity of the intact platelet. Treatment of platelet suspensions with 1 μM PMA for 1 min decreased the basal level of [^{3}H]cAMP (0.038 $\pm$ 0.002% of platelet ^{3}H) by 18 $\pm$ 1% (mean values $\pm$ S.E.M., 8 expts.). The initial rate of accumulation of [^{3}H]cAMP induced by addition of both 0.2 μM PGE_1 and 1 mM IBMX one min after PMA was also diminished (Fig. 6.1.). Thus, PMA inhibited the accumulation of [^{3}H]cAMP observed 15 s after addition of PGE_1 and IBMX by 24 $\pm$ 3% (mean $\pm$ S.E.M., 5 expts.) and that observed after 30 s by 16 $\pm$ 3% (mean $\pm$ S.E.M., 8 expts.). Studies by Murphy (in Williams *et al.*; 1987) showed that [^{3}H]cAMP levels increased more slowly in longer incubations and that, after 1 min, the inhibitory effect of PMA was replaced by stimulation.

Whereas addition of both CP and CPK almost abolished the inhibition of PGE_1-induced [^{3}H]cAMP formation by 10 μM ADP, these additions had only a small effect on the inhibitory action of PMA, which therefore cannot be attributed to the release of platelet ADP (Table 6.1). In further support of this view, the inhibitory action of PMA was observed when the compound was added with PGE_1 and was optimal when added only 30 s before PGE_1 (Fig. 6.2). Inhibition of [^{3}H]cAMP formation by PMA was structurally specific and was not seen with phorbol or 4 α-PDD (Table 6.1). DiC_8, on the other hand, which reproduces many of the effects of PMA on platelets (Lapetina *et al.*, 1985), inhibited [^{3}H]cAMP formation induced by addition of PGE_1 and IBMX (Fig. 6.3). Thus, in platelet

preparations labelled with [^{3}H]adenine and [^{32}P]P_i (Section 2.2.3.), 40 μM diC_8 inhibited [^{3}H]cAMP formation by 28 $\pm$ 9% (mean $\pm$ S.E.M., 4 expts.), whereas 100 nM PMA inhibited by 25 $\pm$ 45 (mean $\pm$ S.E.M., 8 expts.). In contrast, low diC_8 concentrations caused a weak but significant stimulation of [^{3}H]cAMP formation (19 $\pm$ 6% at 1 μM, 4 expts.; $P < 0.05$, paired *t* test) that was rarely seen with PMA.

6.2.2. *Effects of PMA and analogues on the agonist-induced inhibition of [^{3}H]cAMP formation*

Preincubation of platelet with 10 nM - 1 μM PMA diminished the α_2-adrenergic inhibition by epinephrine of the [^{3}H]cAMP accumulation caused by addition of PGE_1 with IBMX (Table 6.1; Fig. 6.2. and Fig. 6.3.). This was only partly due to inhibition of the action of PGE_1 by PMA, as [^{3}H]cAMP formation was considerably greater in the presence of PMA, when epinephrine was added. The latter effect of PMA, which was optimal after about 1.5 min, developed more slowly than its action on PGE_1-induced [^{3}H]cAMP formation (Fig. 6.2.), suggesting a different mechanism of action. In four experiments with platelets labelled with only [^{3}H]adenine (see Section 2.2.2.), the inhibition of PGE_1-induced [^{3}H]cAMP formation by 5 μM epinephrine was reduced from 79 $\pm$ 3% to 22 $\pm$ 5% (means values $\pm$ S.E.M.) after preincubation of the platelets with 1 μM PMA for 1 min. Both the inhibitory action of

epinephrine and the suppression of its effects by PMA were unaffected by CP and CPK, indicating that release of platelet ADP played no role in these phenomena (Table 6.1). Neither phorbol nor 4α-PDD added at 1 μM had any effect on the inhibition of [^{3}H]cAMP formation by epinephrine (Table 6.1) but preincubation of the platelets with 4 - 40 μM diC_8 had effects similar to those of 1 - 100 nM PMA (Fig. 6.3). Higher diC_8 concentrations had non-specific inhibitory effects on [^{3}H]cAMP formation (Fig. 6.3). Epinephrine also inhibited [^{3}H]cAMP accumulation caused by addition of forskolin with IBMX; this effect too was attenuated by PMA (Table 6.2).

In the absence of CP and CPK, addition of 10 μM ADP was almost as effective as 5 μM epinephrine in inhibiting PGE_1-induced [^{3}H]cAMP formation (Table 6.1). However, preincubation of the platelets with 1 μM PMA for 1 min had only a small effect on this action of ADP, reducing the inhibition from 72 ± 4% to 59 ± 6% (mean values ± S.E.M.) in three experiments identical to that shown in Table 6.1. Although this effect was statistically significant ($P < 0.05$, paired t test), PMA appeared to act mainly by inhibiting the action of PGE_1, rather than that of ADP.

High concentrations of thrombin (2 - 5 units/ml) decreased the PGE_1-induced accumulation of [^{3}H]cAMP in platelets incubated with indomethacin, CP and CPK, indicating that thrombin itself inhibited platelet adenylate cyclase activity (Table 6.2). This

effect was also attenuated by preincubation of the platelets with PMA, though not as effectively as was the action of epinephrine. Thus, preincubation of doubly-labelled platelets with 100 nM PMA for 1 min decreased the inhibition of [^{3}H]cAMP formation by 2 units of thrombin/ml from 64 ± 5% to 40 ± 6% (mean values ± S.E.M., 3 expts.), whereas under the same conditions the inhibitory effect of epinephrine was reduced from 67 ± 3% to 29 ± 4% (mean values ± S.E.M.; 7 expts.). In contrast, lower concentrations of thrombin (0.2 units/ml) and high concentrations of PAF (1 μM) or AVP (0.4 μM) had no effect on the accumulation of [^{3}H]cAMP caused by addition of PGE_1 and IBMX, either in the absence or presence of PMA (Table 6.2). Similarly, AVP did not affect [^{3}H]cAMP formation induced by forskolin. These negative results were obtained despite the fact that the same platelets responded to PAF, AVP or low thrombin with a change in shape and some aggregation, when stirred in incubation mixtures containing indomethacin, CP and CPK, but no PGE_1 or IBMX (Fig. 6.4.).

6.2.3. *Relationships between the effects of PMA, diC_8 and physiological stimuli on PKC and their effects on [^{3}H]cAMP formation*

Treatment of ^{32}P-labelled platelets with either PMA or diC_8 caused major increases in ^{32}P incorporation into protein P47 (the principal substrate of PKC in platelets (Nishizuka *et al.*,

1984; Imaoka *et al.*, 1983), into myosin light-chain isoforms (P20a,b) and into proteins of 39 - 41 kDa designated P39-41 (Fig. 6.5). The last normally migrated as 2-3 bands but were compressed into a single band in gels from incubation mixtures containing CPK (unpublished work by M.M.L. Davidson in our laboratory). These phosphorylation reactions were detected with 10 nM PMA or 4 μM diC_8 and were maximal with 1 μM PMA or 100 μM diC_8. With the higher concentrations of PMA or diC_8, increased labelling of many minor polypeptides was also detected (Fig. 6.5). Phorbol had none of these effects. Addition of PGE_1 and IBMX 1 min after PMA or diC_8 did not modify the above phosphorylation reactions during the additional 30 s required for measurement of [^{3}H]cAMP formation (Fig. 6.5.). Incorporation of ^{32}P into protein P47 was utilized as the most sensitive means of quantifying the endogenous activity of PKC in the platelets.

The relationships between the activation of PKC and changes in [^{3}H]cAMP formation were investigated in platelets labelled with both [^{3}H]adenine and [^{32}P]P_i. On addition of increasing concentrations of PMA, a reciprocal relationship was observed between the incorporation of ^{32}P into protein P47 and the inhibition by epinephrine of PGE_1-induced [^{3}H]cAMP formation (Fig. 6.6). As described above, the latter effect consisted of two components (see Fig. 6.2); both showed the same relationship to the phosphorylation of protein P47. Similar results were obtained in platelets treated

with 4-40 μM diC_8 (Fig. 6.6). These findings suggest that the major effects of both PMA and diC_8 on platelet adenylate cyclase are mediated by PKC.

To determine whether activation of PKC by physiological agonists was sufficient to affect [^{3}H]cAMP formation, both were studied in the same platelets and were compared with the actions of PMA (Tables 6.2 and 6.3). In the absence of PGE_1 and IBMX (but with indomethacin, CP and CPK), optimal concentrations of PAF or AVP or a low concentration of thrombin (0.2 units/ml) caused significant increases in the phosphorylation of protein P47 and of the myosin light-chain isoforms (P20a,b), but these effects were much smaller than observed with high thrombin concentrations (2 - 5 units/ml), which also induced detectable increases in the phosphorylation of P39-41 (Fig. 6.7, Table 6.3). Epinephrine on the other hand, did not affect protein phosphorylation in this system. At 100 nM, PMA had effects similar to those of high thrombin concentrations with respect to the phosphorylation of P47 and P39-41, but was less effective with P20a,b (Fig. 6.7), presumably because thrombin stimulates phosphorylation of the latter by both myosin light-chain kinase and PKC (Naka *et al.*, 1983). PMA (100 nM) and thrombin (2 units/ml) did not have additive effects on the phosphorylation of P47 (Table 6.3). Of particular interest was the observation that 10 nM PMA, which had only relatively small effects on [^{3}H]cAMP formation in the presence or absence of epinephrine (Fig. 6.3.), caused an

appreciably greater phosphorylation of P47 than PAF, AVP or low thrombin (Fig. 6.7). This suggests that the latter agonists would be unlikely to modify platelet adenylate cyclase activity by activation of PKC, even in the absence of PGE_1 and IBMX. When PGE_1 and IBMX were present, as was essential for measurement of changes in [^{3}H]cAMP formation, no significant phosphorylation of protein P47 was detected after addition of PAF, AVP or low thrombin, and that caused by high thrombin concentrations was partly inhibited (Table 6.3). Forskolin and IBMX also blocked the phosphorylation of P47 induced by AVP (Table 6.3). Thus, the failure of PAF, AVP and low thrombin to inhibit [^{3}H]cAMP formation in intact platelets cannot be accounted for by an activation of PKC.

6.3. *Discussion*

6.3.1. *Effects of activation of PKC on cAMP formation*

The immediate effect of PMA on [^{3}H]cAMP formation caused by PGE_1 in intact platelets was inhibitory. Even more marked inhibitory effects have been observed with two other activators of adenylate cyclase, forskolin and particularly PGD_2 (Williams *et al.*, 1987). As the initial linear rates of [^{3}H]cAMP accumulation immediately after addition of PGE_1 and IBMX were measured, this effect must reflect an inhibition of adenylate cyclase rather than an activation of cAMP phosphodiesterase. Inhibition by PMA of cAMP

formation induced by PGE_1 (Zucker *et al.*, 1974) or by PGI_2 or forskolin (Ashby *et al.*, 1985) has been observed previously but in the latter study was largely prevented by apyrase, suggesting that it was mediated by the secretion of ADP from the platelet dense granules. However, in the present study, a role for ADP in the inhibition of adenylate cyclase by PMA was excluded by addition of sufficient CP and CPK to reduce the inhibitory effect of a high concentration of exogenous ADP to a very low level. The discrepancy between the present results and an earlier study (Ashby *et al.*, 1985) may be accounted for by major differences in the experimental conditions. Thus, Ashby *et al.* (1985) preincubated platelets with PMA for 10 min rather than 1 min, allowing much more time for secretion of ADP, and then incubated the platelets for a further 5 min with PGI_2 or forskolin in the absence of any inhibitor of cAMP phosphodiesterase. A further indication that differences in the experimental conditions can account for the varied actions of PMA is provided by the findings of Johnson *et al.* (1986) and Bell and Brunton (1987) in S49 lymphoma cells. These investigators demonstrated that the effect of PMA on adenylate cyclase activity was highly dependent on the incubation period, the concentrations of agonist and PMA (Johnson *et al.*, 1986) and the assay temperature (Bell and Brunton, 1987).

The observation that PMA inhibits adenylate cyclase in intact platelets is difficult to reconcile with the enhanced responsiveness of the enzyme to compounds which elevate cAMP in S49

lymphoma cells (Bell *et al.*, 1985; Johnson *et al.*, 1986), cultured bovine adrenal cells (Langlois *et al.*, 1987), Swiss 3T3 cells (Rozengurt *et al.*, 1987) and frog erythrocytes (Yoshimasa *et al.*, 1987). Moreover, Jakobs *et al.* (1985) observed an increased response to PGE_1 or forskolin in the presence of GTP, in membranes isolated from PMA-treated platelets (Jakobs *et al.*, 1985). The effects that Jakobs *et al.* (1985) obtained were attributed to suppression of the inhibitory action of GTP, as a result of phosphorylation of the α_i-subunit of G_i by PKC (Katada *et al*, 1985; Watanabe *et al.*, 1985). As treatment of platelets with PMA did not block the inhibition of membrane adenylate cyclase by a stable GTP analogue, phosphorylation of α_i may act to enhance its GTPase activity and, so facilitate its reassociation with the inhibitory $\beta\gamma$-subunit (Bauer and Jakobs, 1986). Consistent with increased ADP-ribosylation by pertussis toxin after exposure to PMA (Halenda *et al.*, 1986), such a mechanism could lead to an increase but not a decrease in hormone-stimulated cAMP formation, as has also been observed in S49 lymphoma cells (Bell *et al.*, 1985). It follows that, in intact platelets, G_i probably does not exert the tonic inhibitory effect on adenylate cyclase observed in isolated membranes and that PMA is likely to inhibit cAMP formation by effects on components of the adenylate cyclase system other than G_i. Since there is more than one pertussis toxin substrate in platelets and other cells that migrate close to α_i (41 kDa), it is possible that the action of PMA is mediated by

phosphorylation of a G protein distinct from α_i. Likely candidates are the PKC substrates, P39-P41, that were observed in this study. Inhibition of the action of forskolin by PMA suggests that the latter also affects the function of the catalytic subunit of adenylate cyclase or the stimulatory guanine nucleotide-binding protein, G_s, which is required for an optimal forskolin effect (Daly, 1984). The failure of Jakobs *et al.* (1985) and Watanabe *et al.* (1985) to detect any inhibitory effects of PMA or PKC on the adenylate cyclase activities of isolated platelet membranes could reflect selective dephosphorylation of PKC substrates, loss of unidentified soluble factors or a dominant effect of phosphorylation of G_i.

In other cell systems where PMA had a potentiating effect on hormone-stimulated adenylate cyclase activity, this phorbol ester has been shown to have dissimilar sites of action in different cells. For example, activation of PKC by PMA appeared to cause phosphorylation of the catalytic subunit of adenylate cyclase in frog erythrocytes (Yoshimasa *et al.*, 1987), the α_i subunit in S49 lymphoma cells (Johnson *et al.*, 1986) and the β subunit in bovine adrenal cells (Langlois *et al.*, 1987). Instead of postulating that phosphorylation of α_i blocks the ability of this protein to inhibit adenylate cyclase, Johnson *et al.* (1986) and Millar and Rozengurt (1988) have suggested that this covalent modification of α_i may change its function and cause it to

activate the enzyme. However, evidence against a role for α_i in the stimulatory action of PMA is the inability of pertussis toxin to block the effect of PMA (Langlois *et al.*, 1987; Johnson *et al.*, 1986) and the lack of correlation between the amounts of α_i present and the stimulatory action of PMA in 7-day-old cultured adrenal cells (Langlois *et al.*, 1987). Moreover, PMA and pertussis toxin have been shown to inhibit PIP_2 breakdown in polymorphonuclear leukocytes by acting at different sites (Smith *et al.*, 1987). In contrast to the inability of PMA to block the inhibition of adenylate cyclase by GTPγS in human platelet membranes (Bauer and Jakobs, 1986), PMA impaired the coupling of the activated G protein to phospholipase C in polymorphonuclear leukocytes, whereas pertussis toxin interfered with the coupling of the fMLP receptor to the G protein (Smith *et al.*, 1987). In the absence of detailed information on the effects of phosphorylation of specific components of the adenylate cyclase system, indirect effects of PMA are also possible. Thus, Levitzki (1987b) has argued that the evidence for a phosphorylated catalytic unit mediating the stimulatory action of PMA in frog erythrocytes is incomplete and suggests that the effects of PMA could be accounted for by PMA-induced increases in cytoplasmic Ca^{2+}, which then activates a calmodulin-sensitive adenylate cyclase.

In studies in which treatment of cells with PMA has inhibited (Johnson *et al.*, 1986; Nambi *et al.*, 1985; Sugden

et al., 1985; Bell and Brunton, 1987) or desensitized (Kelleher *et al.*, 1984; Sibley *et al.*, 1984) agonist-induced stimulation of adenylate cyclase, the action of PMA has often been attributed to the phosphorylation and consequent inactivation of membrane receptors by PKC. However, since the small decreases in agonist affinity in PMA-treated S49 lymphoma cells do not correlate with the large decreases in functional responsiveness of the hormone-sensitive adenylate cyclase, it is likely that PMA also acts at other sites (Bell and Brunton, 1987). Multiple sites of action of PMA has been reported within a given cell. Bell and Brunton (1987) have shown that PMA induces a number of different effects, some of which are opposing, on the stimulation of adenylate cyclase activity by agents which act on different components of the enzyme in S49 lymphoma cells.

6.3.2. *Relationship between activation of PKC in the platelet and the inhibition of cAMP formation by physiological agonists*

In contrast to the effects of PMA on the activation of adenylate cyclase in intact platelets, the attenuation of the inhibitory effects of epinephrine and thrombin is entirely consistent with previous observations on isolated membranes (Jakobs *et al.*, 1985; Watanabe *et al.*, 1985), and may be accounted for by the phosphorylation of the α_i-subunit of G_i. However, the relative failure of PMA to block the inhibition of [^{3}H]cAMP

formation caused by ADP is anomalous. The inhibition of platelet membrane adenylate cyclase by ADP has been shown to be GTP-dependent (Cooper and Rodbell, 1979; Wakelam *et al.*, 1986) but the effect of pertussis toxin on this is unknown. The present results suggest that the mechanism of inhibition of platelet adenylate cyclase by ADP may differ from that utilized by epinephrine. Bell and Brunton (1987) found that the action of PMA was agonist-specific in S49 lymphoma cells in that PMA enhanced the stimulation of adenylate cyclase by isoproterenol but inhibited the stimulatory action of PGE_1 at 37°C.

The effects of PMA on both the stimulation of platelet cAMP formation by PGE_1 and the inhibition of this by epinephrine were reproduced by diC_8 but not by the biologically inactive PMA analogues, phorbol and 4α-PDD. Moreover, a close relationship was found between these effects and the phosphorylation of the 40-47 kDa platelet substrate of PKC (P47). These observations indicate that PKC mediates these actions of PMA, though there is no reason to believe that P47, which is a soluble protein (Imaoka *et al.*, 1983), itself affects adenylate cyclase activity. Studies on the effects of PMA on protein phosphorylation in intact ^{32}P-labelled platelets have identified many other substrates of PKC, including myosin light chain (Naka *et al.*, 1983) and various transmembrane glycoproteins (Feuerstein *et al.*, 1985; Bourguignon *et al.*, 1985). In the present study, major increases in ^{32}P incorporation

into polypeptides in the 39-41 kDa range (P39-41) were noted. These probably include the 41 kDa α_i-subunit of G_i, which is phosphorylated on incubation of platelet membranes with PKC (Katada *et al.*, 1985). The 41 kDa protein phosphorylated in ^{32}P-labelled platelets exposed to thrombin co-migrates with α_i and binds to an antibody specific to α_i on immunoblots (Crouch and Lapetina, 1988). These workers have suggested that phosphorylation of this protein by PKC plays a role in the desensitization of platelets to thrombin.

In contrast to the above findings, the weak stimulation of [^{3}H]cAMP formation by diC_8 concentrations below those causing inhibition was not associated with phosphorylation of platelet proteins and is unlikely to be mediated by PKC. Ashby *et al.* (1985) observed a more marked stimulation of cAMP formation by 1-oleoyl-2-acetylglycerol, which may represent the same phenomenon, perhaps attributable to a direct perturbation of the membrane bilayer.

The results confirm previous observations that AVP and PAF, which inhibit adenylate cyclase in isolated platelet membranes, do not do so in intact human platelets (Vanderwel *et al.*, 1983; Haslam *et al.*, 1985; Chapter 5). However, the explanation of these findings offered by Jakobs *et al.* (1985), namely that these agonists activate PKC, and thus impair signal transduction by

phosphorylation of G_i, is not supported by our results. Thus, these agonists were only weak activators of PKC under experimental conditions that prevented the formation or action of secondary mediators, such as TxA_2 and ADP. Moreover, in the presence of PGE_1, which is necessary for detection of any inhibition of cAMP formation, AVP and PAF were unable to activate PKC at all. This reflects the well-documented inhibition of phospholipase C activation by agents that increase platelet cAMP (Nishizuka, 1984). More complex results were obtained with thrombin. A low concentration (0.2 units/ml) that causes an optimal GTP-dependent inhibition of adenylate cyclase in membrane preparations (Aktories and Jakobs, 1984), behaved identically to AVP and PAF and did not inhibit [^{3}H]cAMP formation in intact platelets or activate PKC in the presence of PGE_1. Higher thrombin concentrations both inhibited [^{3}H]cAMP formation and activated PKC. The results suggest that this inhibition of adenylate cyclase could consist of two components. First, thrombin may exert a direct effect through G_i (Aktories and Jakobs, 1984), though this may be partially blocked by the simultaneous activation of PKC (Katada *et al.*, 1985), and second, thrombin may inhibit adenylate cyclase through PKC. This would explain why PMA was less effective in blocking inhibition caused by thrombin than that caused by epinephrine.

It has been previously noted that agonists that activate platelet phospholipase C seem unable to inhibit adenylate cyclase in

intact human platelets, despite their ability to do so in membrane preparations (Haslam *et al.*, 1985; Chapter 5). The present results show that this generalization breaks down in the case of high though not low thrombin concentrations, suggesting that the strength of the stimulus may be an important factor in determining the effector system that is activated. In a study by McGowan & Detwiler (1986), this difference was not observed, but chymotrypsin treatment of platelets was found to block the inhibition of cAMP formation by thrombin but not the activation of phospholipase C or PKC. These workers also found that γ-thrombin exerted the same effects as were obtained with low concentrations of α-thrombin in the present study. At least two explanations of the results and of the related findings of McGowan and Detwiler (1986) are possible.

Firstly, distinct receptor subtypes could mediate the stimulation of phospholipase C and inhibition of adenylate cyclase, as suggested for thrombin in human platelets (Holmsen *et al.*, 1981; McGowan and Detwiler, 1986), for muscarinic agonists in a variety of cells (Heller-Brown and Brown, 1984; Ashkenazi *et al.*, 1987) and P_2-purinergic agonists in rat hepatocytes (Okajima *et al.*, 1987). In this case, a mechanism must exist for the selective inactivation of the adenylate cyclase pathway in the intact human platelet. The findings of Murphy *et al.* (1987) on the rapid desensitization of glucagon-stimulated cAMP production in intact hepatocytes after incubation with PMA or agonists that activate

phosphoinositide breakdown supports this hypothesis. These investigators postulated distinct receptors for glucagon-mediated activation of phospholipase C and adenylate cyclase and that activation of the former caused desensitization of the receptors coupled to adenylate cyclase.

Alternatively, a single receptor type could interact with both adenylate cyclase and phospholipase C in isolated membranes, but preferentially with the phospholipase C effector system in the intact cells. There is good evidence that different G proteins can mediate the inhibition of adenylate cyclase and activation of phospholipase C in platelets (Houslay *et al.*, 1986a,b; Grandt *et al.*, 1986; Lapetina 1986; Banga *et al.*, 1988; Chapter 4) but little compelling evidence for distinct receptor subtypes that could mediate these effects (Vanderwel *et al.*, 1983; Haslam *et al.*, 1985; McGowan and Detwiler, 1986; Chapter 5). Thus, in the intact platelet, the receptors for PAF, AVP and thrombin may interact preferentially with the G-protein that activates phospholipase C, so that only when large numbers of receptors are occupied by a full agonist is G_i also activated. This hypothesis is consistent with the present observations and could explain why high thrombin concentrations but neither low concentrations nor γ-thrombin can inhibit adenylate cyclase in intact human platelets. This view is also supported by the finding that PAF does inhibit adenylate cyclase in intact rabbit platelets (Haslam and Vanderwel, 1982), which

possess many more PAF receptors than human platelets (Hwang *et al.*, 1983; Inarrea *et al.*, 1984).

Recent studies by Crouch and Lapetina (1988) on the desensitization of thrombin-induced phospholipase C activation and Ca^{2+} mobilization, suggest that phosphorylation of α_i by PKC mediates this response. Thus, the findings of Jakobs *et al.* (1985) and Crouch and Lapetina (1988) imply that the phosphorylation of α_i by PKC regulates the coupling of receptors to adenylate cyclase and phospholipase C, respectively. However, this hypothesis is not supported by the results of this study nor that of Brass *et al.* (1988). The latter study suggested that the inability of PAF and AVP to decrease cAMP formation in saponin-permeabilized platelets was not due to the failure of these compounds to interact with the G protein thought to be involved in the inhibition of adenylate cyclase as they both decreased the [^{32}P]ADP-ribosylation of a 41 kDa membrane protein. Thus, the mechanism underlying the failure of PAF and AVP to decrease the cAMP formation caused by inhibitory platelet agonists, such as PGE_1, is still unknown. The above findings do not rule out regulation of the adenylate cyclase system by a soluble protein that is retained in saponin-permeabilized cells.

6.4. *Summary*

6.4.1. The immediate and predominant effects of PMA and diC_8 on cAMP formation caused by PGE_1 in human platelets were inhibitory and were attributable to inhibition of adenylate cyclase. Released ADP did not mediate these inhibitory effects as they were not blocked by CP/CPK. Because phosphorylation of $G_{i\alpha}$ by PKC cannot account for an inhibitory action of PMA on adenylate cyclase, other components of this enzyme system must serve as targets of PKC in the platelet.

6.4.2. PMA and diC_8 attenuated the inhibitory action of epinephrine. PMA had less effect on the inhibition of adenylate cyclase by thrombin and almost no effect on decreases in PGE_1-induced cAMP accumulation caused by ADP. The results suggest that these agonists inhibit adenylate cyclase by different mechanisms.

6.4.3. Attenuation of the inhibitory action of epinephrine on PGE_1-stimulated cAMP formation by PMA or diC_8 correlated with increases in the phosphorylation of the major platelet PKC substrate, P47 and of P39-41. The latter may include $G_{i\alpha}$, phosphorylation of which is likely to be responsible for this effect.

6.4.4. PAF, AVP and low thrombin concentrations, all of which inhibit adenylate cyclase in isolated platelet membranes, did not affect [^{3}H]cAMP formation in intact human platelets. However, the activation of PKC by these agonists was insufficient to account for their failure to inhibit [^{3}H]cAMP formation. Moreover, high thrombin concentrations simultaneously activated PKC and inhibited [^{3}H]cAMP formation.

Fig. 6.1. Effect of PMA on the increases in platelet [^{3}H]cAMP caused by PGE_1 and IBMX

Samples of a suspension of platelets labelled with [^{3}H]adenine as described in Section 2.2.3. were first incubated for 5 min with indomethacin, CP and CPK, followed by 1 min with DMSO (●) or 0.1 μM PMA (◆). Basal [^{3}H]cAMP after 1 min exposure to DMSO or PMA and the [^{3}H]cAMP levels 0.25, 0.5 and 1 min after the subsequent addition of 0.2 μM PGE_1 and 1 mM IBMX are shown. All incubations were performed in triplicate. The basal value for [^{3}H]cAMP was subtracted in calculation of the increases shown (mean values ± S.E.D.).

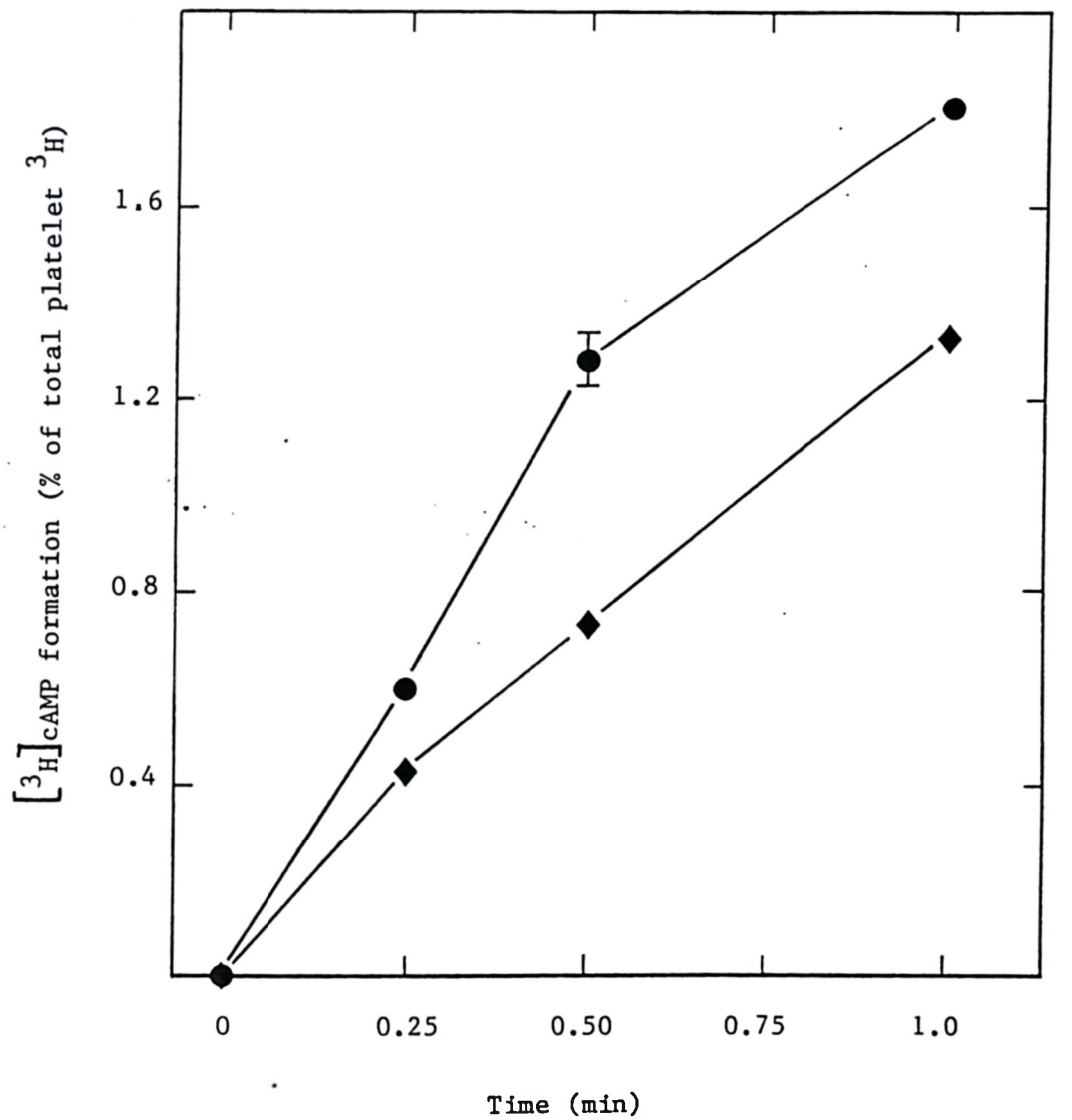

[3H]cAMP formation (% of total platelet 3H)
1.6
1.2
0.8
0.4
0
0.25
0.50
0.75
1.0
Time (min)

Fig. 6.2. Effect of the period of preincubation with PMA on the increases in platelet [^{3}H]cAMP caused by PGE_1 added without or with epinephrine

Samples of a suspension of platelets labelled with [^{3}H]adenine as described in Section 2.2.3. were first incubated for 5 min with indomethacin, CP and CPK. After addition of DMSO (open symbols) or 100 nM PMA (closed symbols), the samples were incubated for a further 0, 0.5, 1.5 or 5 min before [^{3}H]cAMP formation was initiated by addition of 0.2 μM PGE_1 with 1 mM IBMX (○, ●) or of 0.2 μM PGE_1, 5 μM epinephrine and 10 μM propranolol with 1 mM IBMX (△, ▲). Incubations were stopped after 0.5 min by addition of TCA. All were performed in triplicate. The basal values for [^{3}H]cAMP found in the absence of IBMX, PGE_1 or epinephrine at each time point were subtracted from all values shown (mean values ± S.E.D.).

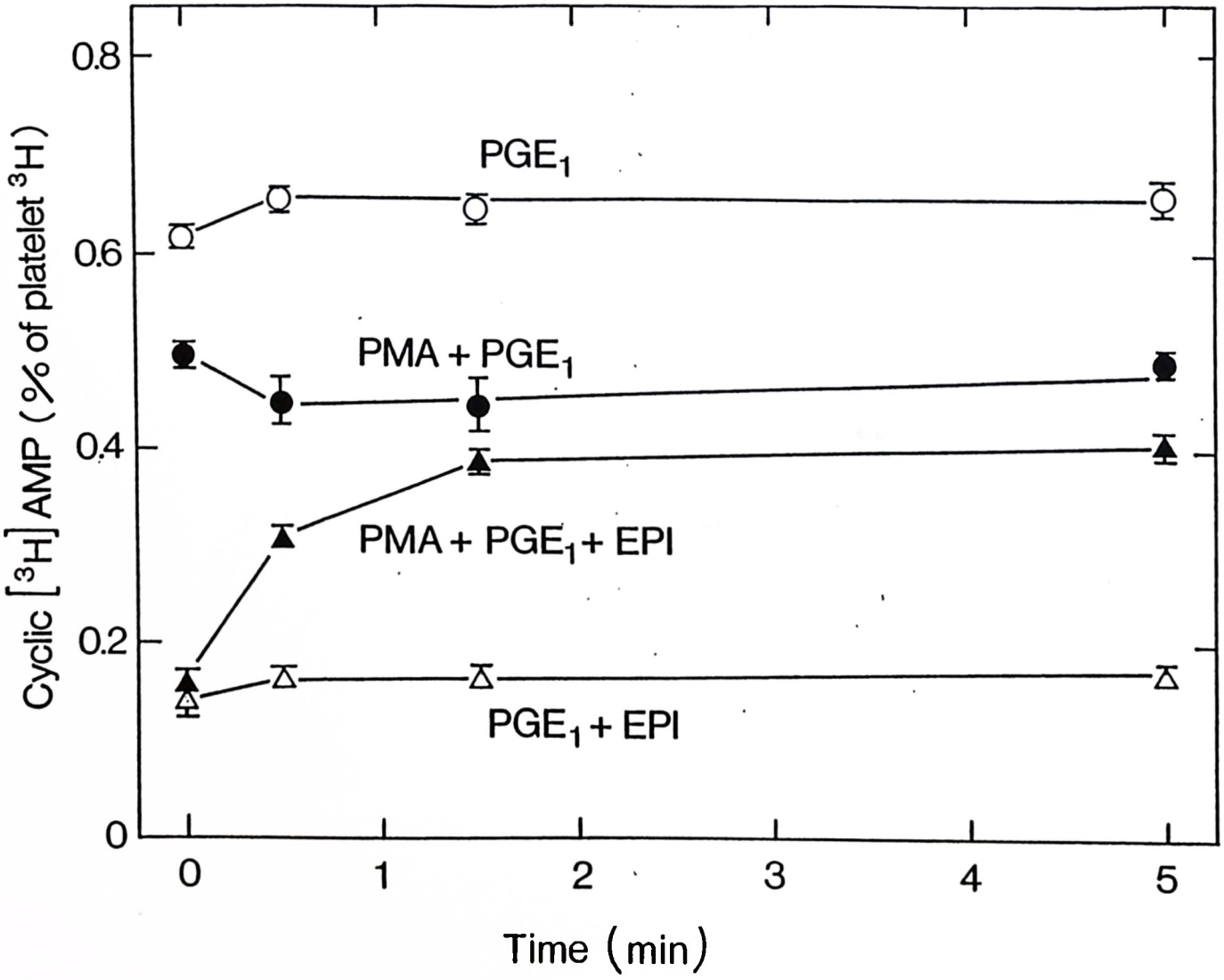

0.8
0.6
0.4
0.2
0
Cyclic [^{3}H] AMP (% of platelet ^{3}H)
PGE$_1$
PMA + PGE$_1$
PMA + PGE$_1$ + EPI
PGE$_1$ + EPI
0
1
2
3
4
5
Time (min)

Fig. 6.3. Effects of different concentrations of PMA and of diC_8 on the increases in platelet [^{3}H]cAMP caused by PGE_1 added without or with epinephrine

Washed platelets were labelled by incubation with both [^{3}H]adenine and [^{32}P]P_i, as described in Section 2.2.4.. Samples were incubated for 5 min with indomethacin, CP and CPK, followed by a 1 min with DMSO (open symbols) or the indicated concentrations of PMA or diC_8 (solid symbols). [^{3}H]cAMP formation was then initiated by addition of 0.2 μM PGE_1 with 1 mM IBMX (○, ●) or of 0.2 μM PGE_1, 5 μM epinephrine and 10 μM propranolol with 1 mM IBMX (△, ▲). Incubations were stopped after 0.5 min by addition of TCA. All were carried out in triplicate. The basal values for [^{3}H]cAMP was subtracted in calculation of the increases shown (mean values ± S.E.D.).

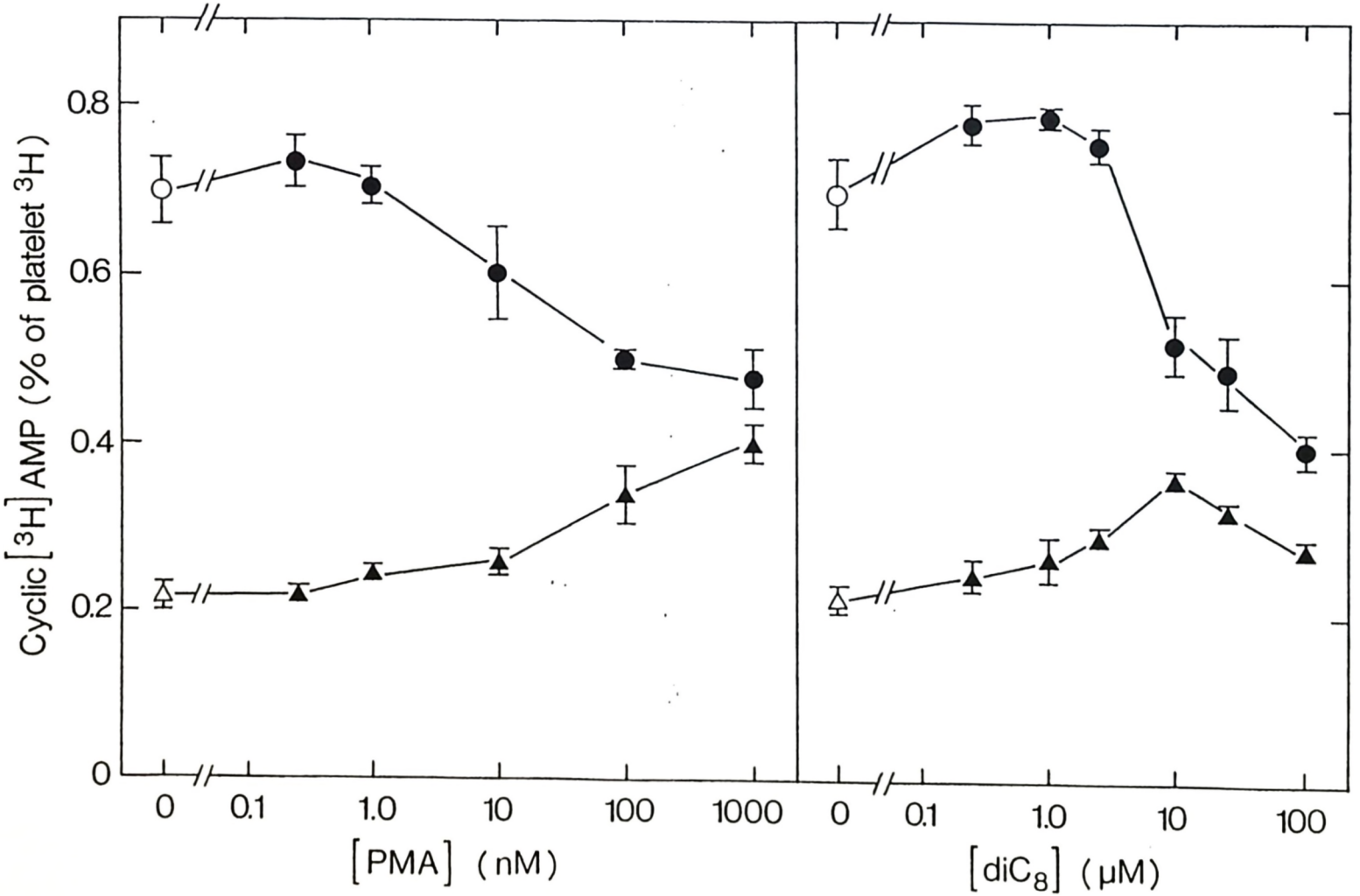

Cyclic [3H] AMP (% of platelet 3H)
0.8
0.6
0.4
0.2
0
0
0.1
1.0
10
100
1000
[PMA] (nM)
0
0.1
1.0
10
100
[diC8] (μM)

Fig. 6.4 Effects of PAF, AVP and a low concentration of thrombin on washed human platelets: induction of shape change and platelet aggregation

Washed human platelets were prepared as in Section 2.2.2. and incubated for 5 min with indomethacin, CP and CPK. Prior to the addition of aggregating agents, platelets were stirred for 2 min and incubated with 0.2 mg of human fibrinogen/ml for 0.5 min. Platelet aggregation was measured turbidometrically as described in Section 2.2.17. The arrows indicate when the following aggregating agents were added: 0.4 μM PAF (a); 0.4 μM AVP; 0.2 units of thrombin/ml (c).

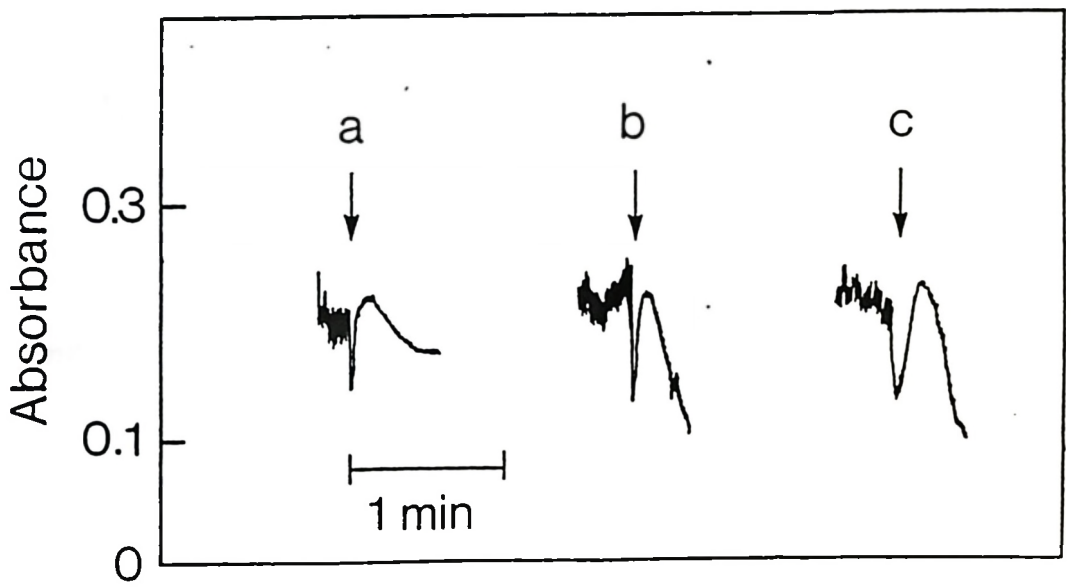
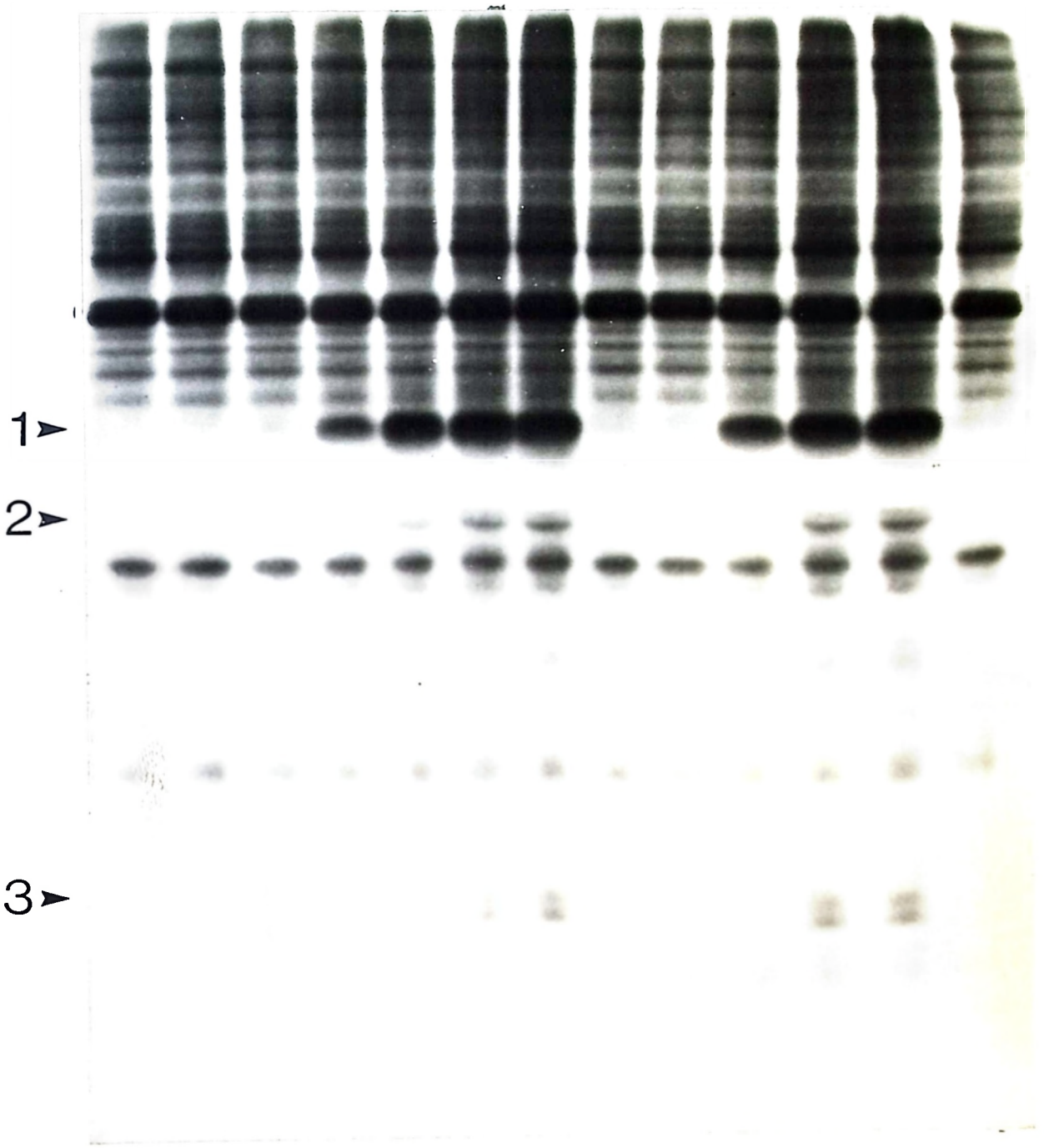


Fig. 6.5. Effects of different concentrations of PMA and of diC_8 on the phosphorylation of proteins in washed platelets

This autoradiograph is from the experiment described in Fig. 6.3. Washed platelets labelled with [^{3}H]adenine and [^{32}P]P_i were incubated for 1 min with DMSO, diC_8 or PMA, followed by 0.5 min with 0.2 μM PGE_1 and 1 mM IBMX. Protein was precipitated with a final concentration of 5% TCA, dissolved in electrophoresis sample buffer and analysed by SDS/polyacrylamide-gel electrophoresis as described in Section 2.2.15. Lanes are as follows: (a) DMSO; (b) 0.4 μM diC_8; (c) 1.0 μM diC_8; (d) 4 μM diC_8; (e) 10 μM diC_8; (f) 40 μM diC_8; (g) 100 μM diC_8; (h) 0.4 nM PMA; (i) 1.0 nM PMA; (j) 10 nM PMA; (k) 100 nM PMA; (l) 1 μM PMA; (m) 100 nM phorbol. The following PKC substrates are indicated: P47 (1); P39-41 (2); P20a,b (3).


a b c d e f g h i j k l m
1
2
3

Endogenous protein kinase C activity
32P in P47 (cpm) (○,●)
0
100
200
300
400
0
0.1
1.0
10
100
1000
[PMA] (nM)
0
0.1
1.0
10
100
[diC8] (μM)
20
40
60
80
Inhibition of cyclic [3H]AMP formation
by epinephrine (%) (◇, ◆)

Fig. 6.7. Comparison of the effects of different aggregating agents and PMA on the phosphorylation of proteins in washed human platelets in the absence of PGE_1 and IBMX

Platelets labelled with both [^{3}H]adenine and [^{32}P]P_i as described in Section 2.2.4. were incubated for 5 min with indomethacin, CP and CPK, followed by 1 min with DMSO or PMA. Aggregating agents were then added and the incubations were terminated after 0.5 min by addition of TCA to give a final concentration of 5%. Precipitated protein was analysed by SDS/polyacrylamide-gel electrophoresis as described in Section 2.2.15. and an autoradiograph of the dried gel is shown. Lanes are as follows: (a) DMSO; (b) 1 μM PAF; (c) 0.4 μM AVP; (d) 0.2 units of thrombin/ml; (e) 2 units of thrombin/ml; (f) 5 units of thrombin/ml; (g) 5 μM epinephrine (with 10 μM propranolol); (h) 10 nM PMA; (i) 100 nM PMA. The following PKC substrates are indicated: P47 (1); P39-41 (2); P20a,b (3).

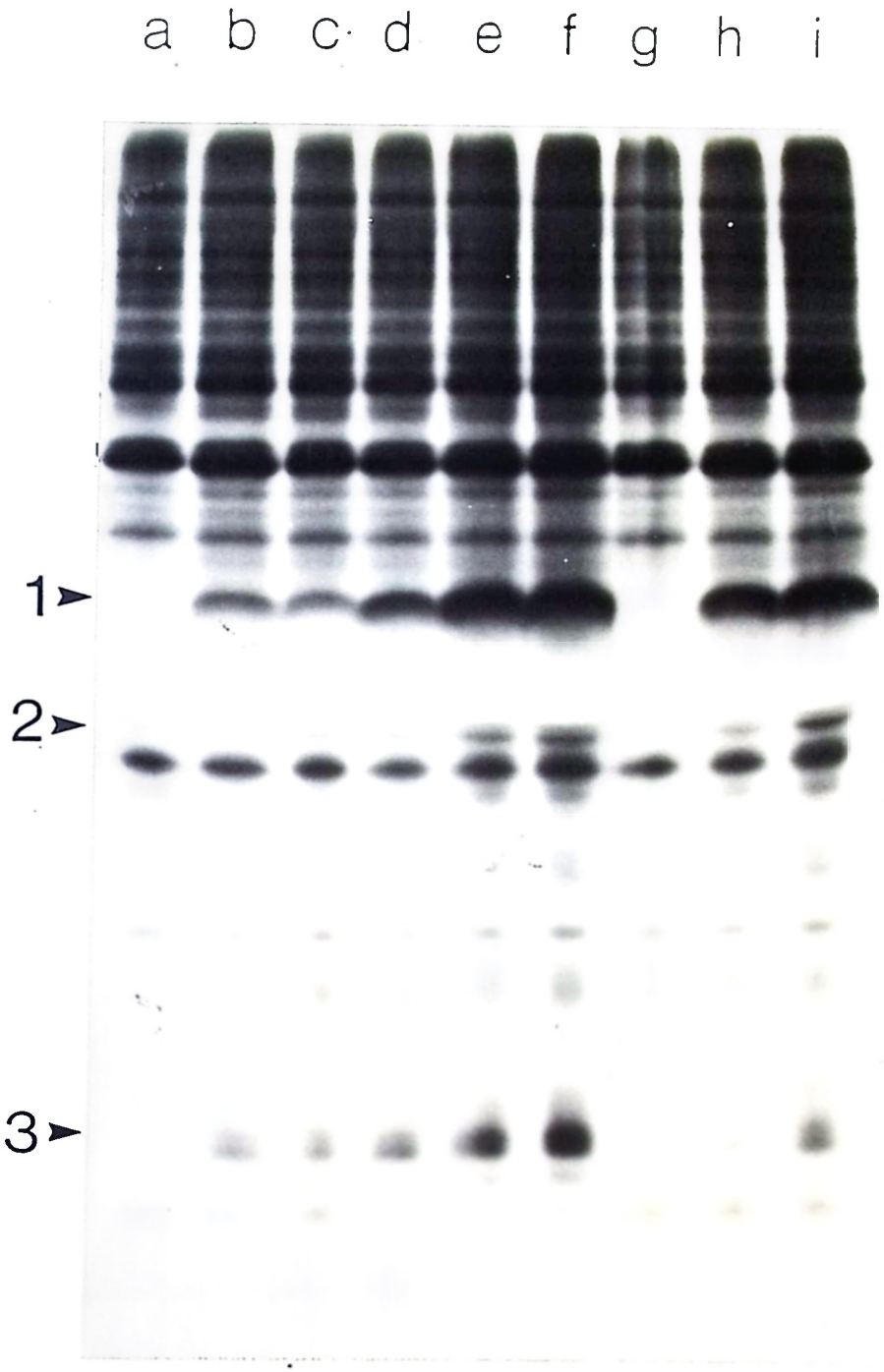

a b c d e f g h i
1
2
3

Table 6.1. Effects of PMA on the formation of [^{3}H]cAMP induced by PGE_1 and on the inhibition of this reaction by epinephrine and by ADP

Samples of a suspension of platelets labelled with [^{3}H]adenine were incubated for 5 min with indomethacin in the absence or presence of CP and CPK as described in Section 2.2.14. and then for a further 1 min with DMSO, 1 μ M PMA, 1 μ M phorbol or 1 μ M 4α-PDD, as indicated. [^{3}H]cAMP formation was then initiated by addition of 0.2 μM PGE_1 and 1 mM IBMX with or without 5 μM epinephrine or 10 μM ADP. Propranolol (10 μM) was included with epinephrine. The incubations were terminated after 0.5 min by addition of TCA. All incubations were performed in triplicate. The basal value for platelet [^{3}H]cAMP was subtracted in calculation of increases (means ± S.E.D.). Percent inhibitions of [^{3}H]cAMP formation by epinephrine or ADP are given (means ± S.E.Q).

6.1.

Initial additions		Additions with PGE_1 and IBMX	Increase in [^{3}H]cAMP (% of total platelet ^{3}H	Inhibition of [^{3}H]cAMP formation (%)
0 min	5 min	6 min		
Indomethacin	DMSO	None	1.266 ± 0.022	-
		Epinephrine	0.196 ± 0.011	84 ± 1
		ADP	0.301 ± 0.011	76 ± 1
	PMA	None	0.718 ± 0.062	-
		Epinephrine	0.465 ± 0.014	35 ± 6
		ADP	0.235 ± 0.014	67 ± 3
Indomethacin + CP + CPK	DMSO	None	1.333 ± 0.013	-
		Epinephrine	0.190 ± 0.002	86 ± 1
		ADP	1.162 ± 0.018	13 ± 2
	PMA	None	0.899 ± 0.032	-
		Epinephrine	0.586 ± 0.017	35 ± 3
	Phorbol	None	1.302 ± 0.060	-
		Epinephrine	0.188 ± 0.002	86 ± 1
	4 αPDD	None	1.282 ± 0.049	-
		Epinephrine	0.177 ± 0.004	86 ± 1

Table 6.2. Effects of different aggregating agents on the increases in platelet [^{3}H]cAMP caused by PGE_1 or by forskolin in the absence and presence of PMA

Washed platelets labelled with both [^{3}H]adenine and [^{32}P]P_i as described in Section 2.2.4. were first incubated for 5 min with indomethacin, CP and CPK, followed by 1 min with DMSO or 100 nM PMA. [^{3}H]cAMP formation was then initiated by addition of 0.2 M PGE_1 with 1 mM IBMX or of 20 M forskolin with 1 mM IBMX at the same time as the aggregating agents indicated. Propranolol (10 μM) was included with epinephrine. After 0.5 min, the incubations were stopped with TCA and [^{3}H]cAMP was determined. All incubations were performed in triplicate. The basal value for [^{3}H]cAMP was subtracted in calculation of the increases shown (means $\pm$ S.E.D). The percent inhibitions of [^{3}H]cAMP formation by the aggregating agents are given (means $\pm$ S.E.Q). Significant effects are indicated (*, $P < 0.05$; **, $P < 0.01$, unpaired t test).

6.2.

Additions		Increase in [^{3}H]cAMP (% of total platelet ^{3}H)	Inhibiton of [^{3}H]cAMP formation (%)
5 min	6 min		
DMSO	PGE_1 + IBMX	0.729 ± 0.020	-
	PGE_1 + IBMX + PAF (1 μM)	0.763 ± 0.017	- 4 ± 4
	PGE_1 + IBMX + AVP (0.4 μM)	0.734 ± 0.022	- 1 ± 4
	PGE_1 + IBMX + Thrombin (0.2 units/ml)	0.659 ± 0.022	10 ± 4
	PGE_1 + IBMX + Thrombin (2 units/ml)	0.241 ± 0.017	67 ± 2**
	PGE_1 + IBMX + Thrombin (5 units/ml)	0.191 ± 0.003	74 ± 1**
	PGE_1 + IBMX + Epinephrine (5 μM)	0.289 ± 0.023	60 ± 3**
	Forskolin + IBMX	0.840 ± 0.009	-
	Forskolin + IBMX + AVP (0.4 μM)	0.823 ± 0.032	2 ± 4
	Forskolin + IBMX + Epinephrine (5 μM)	0.417 ± 0.007	50 ± 1**
PMA	PGE_1 + IBMX	0.585 ± 0.027	-
	PGE_1 + IBMX + PAF (1 μM)	0.662 ± 0.011	-13 ± 6
	PGE_1 + IBMX + AVP (0.4 μM)	0.565 ± 0.035	3 ± 7
	PGE_1 + IBMX + Thrombin (0.2 units/ml)	0.614 ± 0.015	- 5 ± 5
	PGE_1 + IBMX + Thrombin (2 units/ml)	0.337 ± 0.027	42 ± 5**
	PGE_1 + IBMX + Thrombin (5 units/ml)	0.351 ± 0.021	40 ± 4**
	PGE_1 + IBMX + Epinephrine (5 μM)	0.465 ± 0.022	21 ± 5*
	Forskolin + IBMX	0.614 ± 0.034	-
	Forskolin + IBMX + AVP (0.4 μM)	0.530 ± 0.021	14 ± 6
	Forskolin + IBMX + Epinephrine (5 μM)	0.458 ± 0.014	25 ± 5*

Table 6.3. Effects of different aggregating agents and of PMA on the activation of PKC in intact human platelets in the absence and presence of increased [^{3}H]cAMP levels

These results are from the same experiment as that reported in Table 6.2, in which further details are given. Platelet protein was analysed by SDS/polyacrylamide-gel electrophoresis and the ^{32}P incorporated into P47 determined, as a measure of the activation of PKC as described in Section 2.2.15.. Values are means $\pm$ S.E.M. from 3 separate incubations. The percent increases in ^{32}P in P47 relative to the control with no additions other than DMSO are also given (means $\pm$ S.E.Q.). Significant effects are indicated (*, $P < 0.05$; **, $P < 0.01$, unpaired t test).

6.3

Additions		^{32}P in P47 (cpm/sample)	Increase in ^{32}P in P47 (%)
5 min	6 min		
DMSO	None	212 ± 17	-
	PAF (1 μM)	373 ± 30	76 ± 20**
	AVP (0.4 μM)	319 ± 19	51 ± 15*
	Thrombin (0.2 units/ml)	454 ± 16	114 ± 19**
	Thrombin (2 units/ml)	644 ± 14	204 ± 25**
	Thrombin (5 units/ml)	700 ± 66	230 ± 41**
	Epinephrine (5 μM)	227 ± 31	7 ± 17
	PGE_1 + IBMX	178 ± 15	-16 ± 10
	PGE_1 + IBMX + PAF (1 μM)	221 ± 29	4 ± 16
	PGE_1 + IBMX + AVP (0.4 μM)	212 ± 23	0 ± 13
	PGE_1 + IBMX + Thrombin (0.2 units/ml)	247 ± 22	17 ± 14
	PGE_1 + IBMX + Thrombin (2 units/ml)	578 ± 51	173 ± 33**
	PGE_1 + IBMX + Thrombin (5 units/ml)	537 ± 50	153 ± 31**
	PGE_1 + IBMX + Epinephrine (5 μM)	202 ± 14	-5 ± 10
	Forskolin + IBMX	184 ± 18	-13 ± 11
	Forskolin + IBMX + AVP (0.4 μM)	225 ± 27	6 ± 15
	Forskolin + IBMX + Epinephrine (5 μM)	192 ± 7	-9 ± 8
PMA	None	759 ± 27	258 ± 31**
	Thrombin (2 units/ml)	540 + 30	155 ± 20
	PGE_1 + IBMX	684 ± 12	223 ± 26**
	Forskolin + IBMX	576 ± 44	172 ± 30**

Chapter 7

General Discussion

7.1. *Specificity of receptor action*

Individual receptors have often been thought to interact with only one effector system. For example, the well-characterized β, α_2 and α_1 subtypes of adrenergic receptors interact with different effectors. The β-adrenoceptor stimulates adenylate cyclase, the α_2-adrenoceptor inhibits adenylate cyclase and the α_1-adrenoceptor activates phospholipase C (Fain and Garcia-Sainz, 1980). However, it is now clear that a number of receptors are capable of altering the activity of more than one and in some cases multiple signaling systems (reviewed in Limbird, 1988). In platelet membranes, PAF inhibited adenylate cyclase and activated phospholipase C activities by guanine nucleotide-dependent processes. However, the evidence as to whether identical or distinct PAF receptors mediate these effects remains inconclusive. Similar activity ratios for the induction of platelet aggregation and the inhibition of adenylate cyclase by a number of PAF analogues suggests that a single receptor may mediate these actions of PAF. However, the requirement for a higher concentration of PAF to cause activation of phospholipase C than inhibition of adenylate cyclase in rabbit platelet membranes indicates that differences may exist either in the receptor type or in numbers of receptors required to modify these enzyme activities. Thus, it is possible that spare receptors, that can be transiently coupled to the inhibitory pathway of adenylate cyclase, must be

occupied by PAF for optimal activation of phospholipase C. Support for a single receptor mediating these two actions of PAF also comes from the findings of Hwang *et al.* (1983) of a single class of high-affinity binding sites for PAF on rabbit platelet membranes. Coupling of the same receptor to distinct G proteins could be responsible for the observed difference in potency of PAF with respect to these two effector systems. The requirement for a greater concentration of GTP for activation of phospholipase C than for inhibition of adenylate cyclase and the differential sensitivity of these effector systems to pertussis toxin treatment (Chapter 4) certainly suggests that dissimilar G proteins may couple PAF receptors to these enzymes. Interaction of a single receptor type with two different G proteins might be expected to generate two different binding affinities for PAF. Although binding studies in platelets with [^{3}H]PAF have not detected more than one class of specific binding sites, Hwang *et al.* (1988) using PAF antagonists have found that the receptor for PAF on human platelets differs from that on human polymorphonuclear leukocytes. Since Hwang *et al.* (1988) found different pertussis toxin sensitivities of activation of phospholipase C by PAF in human platelets and human polymorphonuclear leukocytes, distinct receptor subtypes for PAF may be coupled to distinct G proteins. Thus, two PAF receptor subtypes may exist in a single species. It is conceivable that two functional receptor subtypes are present on the platelet but that one is present in numbers too small to be readily detected in binding assays. This

problem can only be resolved by the discovery of PAF antagonists that show specificity in blocking inhibition of adenylate cyclase or activation of phospholipase C.

Studies designed to characterize receptors for other agonists that both inhibit adenylate cyclase and activate phospholipase C in platelets have also been carried out. In the human platelet, both AVP and thrombin receptors couple to these two signaling mechanisms. Inhibition of adenylate cyclase and induction of platelet aggregation (i.e. activation of phospholipase C) by analogues of AVP show the same structure-activity relationships, though higher concentrations of agonist are required for aggregation (Vanderwel *et al.*, 1983). Identical pA_2 values for a V_1 receptor antagonist in blocking AVP-induced platelet aggregation and inhibition of adenylate cyclase have strongly suggested that very similar receptors of the V_1 type couple with phospholipase C and adenylate cyclase in the platelet (Vanderwel *et al.*, 1983). With the use of agonists and antagonists of AVP that can discriminate between the V_1 receptors in the hepatic (V_{1h}) and anterior pituitary (V_{1a}) membranes, Launay *et al.* (1987) has demonstrated that shape change and aggregation of human platelets by AVP are mediated by V_{1a} receptors. It remains to be determined whether the inhibition of adenylate cyclase by AVP is also mediated by the V_{1a} receptor subtype or by a different receptor subtype that was not detected by the antagonist used in the study by Vanderwel *et al.* (1983).

There is evidence that platelets possess at least two thrombin receptor subtypes. McGowen and Detwiler (1986) found that α-chymotrypsin treatment of human platelets separates the actions of α-thrombin on adenylate cyclase and phospholipase C activities. In chymotrypsin-treated human platelets, α-thrombin was no longer capable of inhibiting adenylate cyclase but still retained its ability to cause platelet activation. Moreover, the actions of α-thrombin in chymotrypsin-treated human platelets were the same as those of γ-thrombin on untreated platelets. These investigators concluded that two receptors or coupling mechanisms for thrombin exist in human platelets and that they interact with different effector systems.

Attempts to determine if a single receptor type or distinct receptors couple to inhibition of adenylate cyclase and activation of phospholipase C in cells other than the platelet have also yielded conflicting results. Differences in the specificity of analogues of ATP and in the actions of pertussis toxin on these two effector systems suggest that distinct purinergic receptors and possibly distinct G proteins couple to the inhibition of adenylate cyclase and activation of phospholipase C in rat hepatocytes (Okajima *et al.*, 1987). Studies of the action of muscarinic agonists on cAMP formation and PI metabolism in embryonic chick heart cells, under very similar experimental conditions, revealed differences in potency, agonist specificity and binding properties of the receptors

coupled to these two effector systems (Heller Brown and Brown, 1984). As with PAF and rabbit platelet membranes, much higher concentrations of muscarinic agonists were required to activate PI metabolism than to decrease cAMP formation under similar experimental conditions. However, it was not conclusively established that distinct muscarinic receptors were involved, as the results could also be accounted for by a difference in the efficiency with which the receptors are coupled to the two effector systems (Heller Brown and Brown, 1984). Support for this hypothesis came from the study of Ashkenazi *et al.* (1987) who assayed the action of muscarinic agonists and antagonists on adenylate cyclase and phospholipase C activities in Chinese hamster ovary cells expressing recombinant M2 muscarinic receptors. The much higher concentrations of carbachol required to activate phospholipase C than to inhibit adenylate cyclase and the higher sensitivity of the former enzyme to the number of M2 receptors suggest that spare receptors exist for the inhibition of adenylate cyclase by carbachol. Pertussis toxin treatment was also much more efficient in uncoupling the M2 receptors from adenylate cyclase than from phospholipase C. A more recent study by Peralta *et al.* (1988) in which four distinct subtypes of the human muscarinic acetylcholine receptor were transfected into human embryonic kidney cells has shown that distinct receptor subtypes can mediate the inhibition of adenylate cyclase and activation of phospholipase C by muscarinic agonists. However, these investigators also found that inhibition of adenylate cyclase required much lower

concentrations of agonists than activation of phospholipase C. Since portions of the cytoplasmic domain of the muscarinic receptor subtypes coupled to the same effector were homologous, it is possible that these amino acid sequences govern the specificity of receptor-G protein coupling to appropriate effectors (Peralta *et al.*, 1988), as has been found with the human β_2 adrenoceptor (O'Dowd *et al.*, 1988).

These precedents indicate that if specific antagonists for PAF receptor subtypes with distinct functions are not found, further progress may depend on the cloning and expression of PAF receptors. Monoclonal antibodies to PAF receptor may also, when they become available, permit distinction between PAF receptors mediating the inhibition of adenylate cyclase and activation of phospholipase C.

7.2. *Specificity of G protein action*

The discovery of multiple guanine nucleotide-dependent mechanisms involved in signal transduction, together with the identification of increasing numbers of G proteins, has made it difficult to determine which G protein(s) couple Ca^{2+}-mobilizing receptors to phospholipase C. To date, there are four distinct species of $G_{s\alpha}$ (Robishaw *et al.*, 1986; Bray *et al.*, 1986), four distinct pertussis toxin substrates (Jones and Reed, 1987; Itoh *et al.*, 1988), a G protein that probably interacts

with neither toxin (G_z) (Fong *et al.*, 1988), ras p21 proteins (Wakelam *et al.*, 1986; Fleishman *et al.*, 1986), $G_p/G_{\alpha 25}$ (Evans *et al.*, 1986; Waldo *et al.*, 1987), ADP-ribosylation factor (Kahn and Gilman, 1986), a number of low molecular mass G_n proteins (Bhullar and Haslam, 1987; Lapetina and Reep, 1987; Bokoch and Parkos, 1988), botulinum toxin substrates (Ohashi *et al.*, 1987; Aktories and Frevert, 1987; Rosener *et al.*, 1987; Aktories *et al.*, 1987) and numerous low molecular mass GTP-binding proteins (e.g. ral, rho, and rab variants) identified in cDNA libraries though limited homology with ras gene products (Chardin, 1988). Immunological methods and cDNA sequencing studies also indicate that heterogeneity exists amongst the β and γ subunits (reviewed in Gilman, 1987; Neer and Clapham, 1988). Besides adenylate cyclase and phospholipase C, G proteins have also been reported to regulate PLA_2 (Jelsema and Axelrod, 1987), phospholipase D (reviewed in Exton, 1988), brain cAMP-dependent phosphodiesterase (Asano *et al.*, 1986), K^+ channels and voltage-sensitive Ca^{2+} channels (reviewed in Brown and Birnbaumer, 1988). Furthermore, receptors which inhibit adenylate cyclase may couple to a number of other signaling systems, causing activation of Na^+/H^+ exchange, increased K^+ conductance and attenuation of voltage-sensitive Ca^{2+} channels (reviewed in Limbird, 1988). Thus, it is possible that particular combinations of the distinct α, β and γ subunits of the G protein determine receptor and effector interactions.

In this thesis, a number of differences were noted in the inhibition of adenylate cyclase and activation of phospholipase C by agonists, suggesting that more than one G protein may mediate receptor coupling to these two effectors. In the dual assay system, PAF and thrombin had effects on both enzymes, whereas epinephrine acted solely as an inhibitor of adenylate cyclase. Since epinephrine inhibited adenylate cyclase via a pertussis toxin-sensitive G_i-related protein and did not activate phospholipase C, the same G_i-related protein is unlikely to couple thrombin and PAF receptors to the latter enzyme. Roles for distinct G proteins are also supported by the finding that maximal activation of phospholipase C by PAF required a higher GTP concentration than the inhibition of adenylate cyclase by this agonist. Differences in monovalent cation requirements were also noted for agonist effects on these two enzymes. The inhibition of adenylate cyclase by PAF was greatest in the presence of NaCl, whereas activation of phospholipase C by PAF was more marked in a KCl-containing medium. Since the actions of NaCl in stimulating adenylate cyclase and in potentiating the inhibitory action of PAF were dependent on GTP (Chapter 3), these differences could be explained by G proteins with different monovalent cation requirements. Alternatively, Na^+ may interact with a component that is separate from the G protein, as suggested by target size analysis of opioid receptors (Ott *et al.*, 1988). The difference in the sensitivities of the inhibition of adenylate cyclase and activation of phospholipase C to pertussis toxin also

suggests that distinct G proteins may be involved. As also observed by Banga *et al.* (1988) with thrombin, the inhibition of adenylate cyclase by PAF was more susceptible to inactivation by pertussis toxin than was the activation of phospholipase C by this agonist. However, it is necessary to be cautious in interpreting this data because of the incomplete ADP-ribosylation of platelet pertussis toxin substrates and the possibility that the amounts required to mediate the two effects studied are different.

The studies in this thesis and in other laboratories designed to identify the G protein coupling Ca^{2+}-mobilizing receptors to phospholipase C have shown that not only do pertussis toxin-sensitive and insensitive G proteins participate but that multiple pertussis toxin substrates also exist. Indeed, the G protein thought to mediate inhibition of adenylate cyclase (G_i) has not yet been clearly identified with any one of the three proteins recognized from their cDNA clones as $G_{i1\alpha}$, $G_{i2\alpha}$ and $G_{i3\alpha}$ (reviewed in Lochrie and Simon, 1988). Thus, the specific pertussis toxin substrate(s) coupling receptors to either adenylate cyclase or phospholipase C are presently unknown and many of the earlier studies, the interpretation of which was based on the presumed existence of only one pertussis toxin substrate, will need to be re-examined. It cannot, however, be ruled out that G protein-effector interactions may be relatively non-specific, as already suggested for the platelet (Brass *et al.*, 1988). In this

connection, Yatani *et al.* (1988) recently demonstrated that all three G_i-related proteins were capable of opening atrial K^+ channels. Further work will thus be necessary to determine the identity and function of the three pertussis toxin substrates detected in rabbit platelet membranes in the present study. With the use of specific polyclonal antibodies to the different G_i-related proteins, the major and minor pertussis toxin substrates in human platelets have been identified as $G_{i2\alpha}$ and possibly $G_{i1\alpha}$, respectively (Nagata *et al.*, 1988). Using the same methodology, it should be possible to identify the three pertussis toxin substrates in rabbit platelet membranes as $G_{i1\alpha}$, $G_{i2\alpha}$, $G_{i3\alpha}$ or $G_{0\alpha}$. Alternately, amino acid sequencing of proteolytic fragments of pertussis toxin substrates excised from 2-dimensional SDS polyacrylamide gels (Kennedy *et al.*, 1988) should permit their identification. To determine which G proteins mediate the pertussis toxin-sensitive components of the inhibition of adenylate cyclase and stimulation of phospholipase C by PAF, pertussis toxin or NEM-treated rabbit platelet membranes could be reconstituted with specific recombinant G proteins. The dual assay system will also be useful for comparison of the actions of a number of other agents on both effector systems and has the potential for uncovering new regulatory factors that affect the hormonal inhibition of adenylate cyclase and/or the activation of phospholipase C.

7.3. *Failure of some agonists that activate phospholipase C to decrease PGE_1-stimulated cAMP formation in intact human platelets*

Examination of the effects of a number of stimulatory agonists on intact human platelets has shown that they are either potent inhibitors of adenylate cyclase or powerful stimulators of phospholipase C but, with the exception of high concentrations of thrombin, not both (Haslam *et al.*, 1985; Brass *et al.*, 1988; this thesis). For example, agonists such as ADP and epinephrine, which have little or no effect on phospholipase C activity (Fisher *et al.*, 1985; Siess *et al.*, 1984), decrease cAMP formation in the intact platelet (Robison *et al.*, 1969; Haslam, 1975), whereas agonists such as PAF, AVP and stable prostaglandin endoperoxides, which activate phospholipase C (Siess *et al.*, 1984; 1986; 1985), have no effect on cAMP formation caused by PGE_1 in the intact human platelet (Chapter 5, 6; Best *et al.*, 1979). This phenomenon could not be explained by any of the hypotheses tested in this thesis (Chapters 5 and 6), including that of Jakobs *et al.* (1985), who postulated that receptor coupling to the inhibition of adenylate cyclase was blocked by phosphorylation of $G_{i\alpha}$, when PKC was activated as a result of the stimulation of phospholipase C. Since PAF does decrease the cAMP formation caused by PGE_1 in intact rabbit platelets (Haslam and Vanderwel, 1982; Chapter 5) and rabbit platelets contain greater numbers of high-affinity binding sites for

[^{3}H]PAF than human platelets (Inarrea *et al.*, 1984), the difference in the ability of PAF to suppress cAMP formation in these two species could be a consequence of a difference in receptor number. Thus, in the intact human platelet, all occupied PAF receptors may couple to phospholipase C and only after removal of an unidentified constraint during preparation of membranes may some of these receptors or other latent PAF receptors become available for coupling to adenylate cyclase. This constraint is likely to reflect the presence of an inhibitor of G_i-adenylate cyclase coupling in the intact human platelet which is removed in the process of preparing platelet particulate fractions. The inability of PAF and AVP to decrease cAMP formation caused by PGI_2 in saponin-permeabilized platelets (Brass *et al.*, 1988), suggests that a low molecular mass compound present in the platelet cytosol is unlikely to be involved. However, since the degree of permeabilization caused by saponin depends critically on the saponin concentration (Brass and Joseph, 1985; Authi *et al.*, 1986), electropermeabilized human platelets which reproducibly release 80% of low molecular mass compounds without loss of lactate dehydrogenase (Haslam and Davidson, 1984a), should be assayed for decreases in PGE_1-stimulated cAMP formation caused by PAF. To determine whether a soluble inhibitor protein is involved, the inhibition of adenylate cyclase by PAF could be assayed in the presence of concentrated dialysed platelet cytosol. The cytosolic protein conferring inhibitory activity could then be purified and

reconstituted with the other components of the inhibitory pathway of adenylate cyclase in phospholipid vesicles to determine its mechanism of action.

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