REGULATION OF PLATELET FUNCTION BY CYTOSOLIC FREE CALCIUM AND CYCLIC AMP

A thesis submitted to the University of Glasgow in candidature for the degree of Doctor of Philosophy in the Faculty of Science.

by

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List of Contents

Much of the information presented in this thesis has been reported in the following publications:

1. Pollock, W.K., Bushfield M., Shaw A.M. & MacIntyre D.E. (1984) Agonist-specific desensitization of human platelet phosphoinositide metabolism and changes in cytosolic calcium. Proc. 9th IUPHAR., 582P. (Abstract).

2. Bushfield M., McNicol A. & MacIntyre D.E. (1985). Inhibition of PAF-induced human platelet activation by PGD_2: differential sensitivity of platelet transduction processes and functional responses to inhibition by cAMP. Biochem. J., 232: 267-271.

3. Bushfield M., MacIntyre D.E., MacMillan L.J. & McNicol A. (1985 Role of phosphoinositide hydrolysis and Ca^{2+}_i in potentiation of platelet activation by adrenaline. Thrombosis Haemostas., 54: 0669 (Abstract).

4. Bushfield M., McNicol A. & MacIntyre D.E. (1985) Differential inhibition of platelet receptor-activated functional responses and transduction processes by cAMP. Br. J. Pharmacol., 86: 577P (Abstract).

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Differential effects of phorbol 12-myristate 13-acetate on PGD_2- and PGI_2-induced cyclic AMP formation in platelets.
Br. J. Pharmacol. (in press) (Abstract).

13. Bushfield M., McNicol, A. & MacIntyre D.E. (1986).
Possible mechanisms of the potentiation of blood platelet activation by adrenaline.
Biochem. J. (in press).

14. MacIntyre D.E., Bushfield M., MacMillan L.J., Moffat K.J., Murdoch F.A., Thompson A., Rossi A.G. & McNicol A. (1986).
Receptors and receptor mechanisms of endogenous platelet stimulatory and inhibitory mediators.
Agents and Actions (in press).

15. MacIntyre D.E., Bushfield M., MacMillan L., Moffat K., Murdoch F., Thompson A., Rossi A. & McNicol A. (1986).
Platelet receptors for endogenous stimulatory and inhibitory mediators.
Proceedings of Cologne Atherosclerosis Conference (in press) (Abstract).

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Synergistic activation of human platelets in whole blood
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Abbreviations

The abbreviations used throughout this thesis are those recognised by the Biochemical Journal. Some of the commonly used abbreviations are listed below.

DTS	-	dense tubular system
SCCS	-	surface-connected cannalicular system
5HT	-	5 hydroxytryptamine; serotonin
AMP	-	adenosine 5' monophosphate
ADP	-	adenosine 5' diphosphate
ATP	-	adenosine 5' triphosphate
cyclic AMP	-	adenosine 3'5' cyclic phosphate
GTP	-	guanosine 5' triphosphate
cyclic GMP	-	guanosine 3'5' cyclic phosphate
PG	-	prostaglandin
Tx	-	thromboxane
PAF	-	platelet-activating factor; acetyl glyceryl ether phosphoryl choline
DG	-	1,2-diacylglycerol
Ptd Ins	-	phosphatidyl inositol
PIP	-	phosphatidyl inositol 4-phosphate
PIP_2	-	phosphatidyl inositol 4,5-bisphosphate
Ptd OH	-	phosphatidic acid
OAG	-	1-oleoyl 2-acetyl glycerol
PMA	-	phorbol 12-myristate 13-acetate
4α-PDD	-	4 alpha phorbol 12, 13-didecanoate
P_i	-	orthophosphate

$[Ca^{2+}]_i$	-	cytosolic free calcium ion concentration
$[Ca^{2+}]_o$	-	extracellular calcium concentration
G protein	-	guanine nucleotide-binding regulatory protein
Kd	-	dissociation constant
EC_{50}	-	agonist concentration inducing 50% of maximum response
I_{50}	-	antagonist concentration causing 50% inhibition of a given response
EGTA	-	ethylene glycol bis (β-amino ethyl ether)-tetraacetic acid
DMSO	-	dimethyl sulphoxide
TCA	-	trichloroacetic acid
Mr	-	relative molecular mass
Quin 2	-	2-[(2-bis-[Carboxymethyl]amino-5-methyl-phenoxy)methyl]-6-methoxy-8-bis [carboxymethyl] aminoquinoline
Quin 2 AM	-	Quin 2-acetoxymethyl ester

Summary

Human blood platelet responsiveness can be modulated by a variety of stimulatory and inhibitory agonists. These agents are recognised by specific receptors which presumably are situated on the outer surface of the platelet plasma membrane. Receptors have been characterised for a number of endogenous compounds including adrenaline, ADP, vasopressin, platelet-activating factor, PGI_2, PGD_2 and adenosine. Platelets are electrically non-excitable, hence, there must exist transduction processes which link events at the cell surface (i.e. agonist-receptor interaction) to the key intracellular reactions which control cellular responsiveness.

In platelets, as in many other cell types, it has been established that inositol phospholipid hydrolysis serves as one of the transduction processes that link stimulatory receptor occupancy to cellular activation. Phospholipase C-catalysed hydrolysis of phosphatidylinositol 4,5-bisphosphate yields 1,2-diacylglycerol, an activator of protein kinase C, and inositol (1,4,5) trisphosphate which can mobilise calcium ions from intracellular stores. Inositol phospholipid hydrolysis may also be involved in the agonist-induced influx of calcium ions which also occurs in platelets. Inhibition of platelet reactivity is associated with agonist-induced stimulation of adenylate cyclase activity and increased intracellular levels of cyclic AMP. Cyclic AMP, via activation of cyclic AMP-dependent protein kinase, can modify a variety of platelet intracellular reactions.

In vivo platelets may be exposed to combinations of stimulatory and/or inhibitory agonists. Hence, the phenomena of synergism and desensitization, which have been demonstrated in vitro, may occur in vivo and may be of major importance in haemostasis and in the pathogenesis of occlusive vascular disease. This study examined the effects of agonists, alone and in combination, on platelet responsiveness and the putative underlying transduction processes of inositol phospholipid hydrolysis, elevation of cytosolic free calcium concentration $\left[Ca^{2+}\right]_i$) and cyclic AMP concentration. Platelet responsiveness was monitored by measuring (a) shape change; (b) aggregation in whole blood (by a single platelet counting method); (c) aggregation in platelet-rich plasma (by two methods: turbidometrically and by counting single platelets); (d) aggregation in gel-filtered platelets and (e) release of dense granule constituents.

Stimulation of inositol phospholipid metabolism was assessed as $\left[^{32}P\right]$-Ptd OH formation in platelets prelabelled with $\left[^{32}P\right]$-P_i. Changes in $\left[Ca^{2+}\right]_i$ were measured using the fluorescent calcium-indicator dye Quin 2 and cyclic AMP levels were measured by radioimmunoassay.

1. In platelet-rich plasma, the single platelet counting method provided a much more sensitive index of aggregation than did the measurement of changes in light transmission in an aggregometer.

2. PAF and vasopressin induced concentration-dependent platelet activation, $[^{32}P]$-Ptd OH formation and elevation of $[Ca^{2+}]_i$. ADP-induced platelet activation was associated with elevation of $[Ca^{2+}]_i$ but not $[^{32}P]$-Ptd OH formation. Adrenaline also caused platelet activation but had no direct effect on $[Ca^{2+}]_i$ or $[^{32}P]$-Ptd OH levels.

3. Adrenaline potentiated ADP-, vasopressin- and PAF-induced platelet activation and elevation of $[Ca^{2+}]_i$. Agonist (vasopressin)-induced $[^{32}P]$-Ptd OH formation was also potentiated.

4. Potentiation of platelet activation by adrenaline may be mediated by enhanced inositol phospholipid hydrolysis and elevation of $[Ca^{2+}]_i$.

5. PGI_2, PGE_1, 6-keto-PGE_1, PGD_2 and adenosine induced concentration-dependent elevations in platelet cyclic AMP content.

6. PAF-induced functional platelet responses and transduction processes show differential sensitivity to inhibition by PGD_2 and PGI_2. A doubling in the platelet cyclic AMP content resulted in abolition of PAF-induced aggregation and ATP secretion, whereas maximal inhibition of shape change, $[^{32}P]$-Ptd OH formation and elevation of $[Ca^{2+}]_i$ required a greater than ten-fold elevation in cyclic AMP. This differential sensitivity of the various responses to inhibition by cyclic AMP suggests that the mechanisms underlying inhibition of PAF-induced aggregation and ATP secretion differ from those underlying shape change. Thus a major component of cyclic AMP-dependent inhibition of PAF-induced aggregation and ATP

secretion is mediated by suppression of certain components of the activation process that occur distal to the formation of diacylglycerol or elevation of $[Ca^{2+}]_i$.

7. Inhibitors of adenylate cyclase (adrenaline, ADP) but not vasopressin or PAF, prevented elevation of cyclic AMP content and inhibition of platelet responsiveness and transduction processes by adenylate cyclase stimulants (PGI_2, PGD_2) but not by other means (e.g. inhibition of phosphodiesterase activity).

8. Activation of protein kinase C by PMA directly inhibited PGD_2- but not PGI_2-, PGE_1-, 6-keto-PGE_1- or adenosine-induced cyclic AMP formation. PMA had no effect on the inhibition of cyclic AMP formation by adrenaline.

9. A preliminary study confirmed the presence of cyclic AMP-dependent protein kinase activity in crude platelet soluble and particulate fractions. Measurement of this enzyme activity may provide a sensitive index of the effects of agonists on cyclic AMP metabolism in platelets.

This study has examined a number of questions in relation to the control of platelet reactivity in the presence of multiple stimulatory or inhibitory agonists. A detailed understanding of the mechanisms which mediate responsiveness under these conditions may facilitate the development of therapeutic agents to specifically modulate platelet reactivity in vivo.

INTRODUCTION

I. Platelet formation

Since their discovery over 100 years ago (Bizzozero, 1882), blood platelets have been shown to play an essential role in haemostasis and thrombosis by virtue of their ability to adhere to damaged endothelium or foreign surfaces and initiate various processes leading to formation of a haemostatic plug. Platelets have their origin in megakaryocytes which follow a unique pattern of proliferation and maturation. Committed megakaryocytic stem cells develop from the common blood cell precursors, pluripotential stem cells, in the bone marrow. From these are derived the diploid megakaryoblast precursors which do not undergo further cell division (Feinendegen et al., 1962). Endomitosis occurs to give the morphologically recognisable cells of the megakaryocyte series. Megakaryocytes develop in three stages: (1) megakaryoblast, (2) basophil megakaryocyte and then (3) granular megakaryocyte. The intense biosynthetic activity during maturation is accompanied by the development of a complex demarcation membrane system throughout the cytosol (Pennington, 1981). When fully developed, the megakaryocytes seek out the marrow sinusoids. Between 20% and 50% of mature megakaryocytes pass through the sinusoids into the circulation whilst the remainder discharge their cytosol into the sinusoids and are fragmented in the flowing blood. The mature megakaryocytes are carried to the microcirculation of the lung where they are then fragmented and discharge their cytoplasm. Small megakaryocytic fractions then take on the regular discoid form of platelets, probably as a result of the orderly rearrangement of the marginal microtubular bundle (Pennington, 1981). The

normal circulating platelet count is around 2-4 x 10^8 per ml and the turnover rate is approximately 3.5 x 10^7 per ml per day (Harker & Finch, 1969). The platelet life-span is usually 7-10 days, spent platelets being removed by sequestration in the liver and spleen (Gordon & Milner, 1976).

II. Platelet structure

Platelets normally circulate as anucleate biconvex discs around 2-3 μm in diameter. The main structural features of quiescent platelets are illustrated schematically in Figure 1. The plasma membrane is rich in glycoproteins which make up the 'glycocalyx'. This appears as a fuzzy surface coat in electron micrographs. Immediately below the plasma membrane in the equatorial plane lies a bundle of microtubules which form a ring round the platelet. These have a cytoskeletal function to maintain the characteristic discoid shape of quiescent platelets (Gordon, 1981). In direct continuity with the plasma membrane is the surface-connected cannalicular system (SCCS). This series of invaginations in the platelet surface greatly increases the platelet surface area. Platelet granule contents may be secreted into the SCCS by exocytosis before diffusing to the external medium. Plasma-borne substances may also enter the SCCS and be transferred to the cytoplasm and granules (White, 1970). The dense tubular system (DTS), immediately associated with the SCCS, contains calcium storage vesicles (White, 1973) and may be the main site of arachidonic acid metabolism in the platelet (Gerrard *et al.*, 1981).

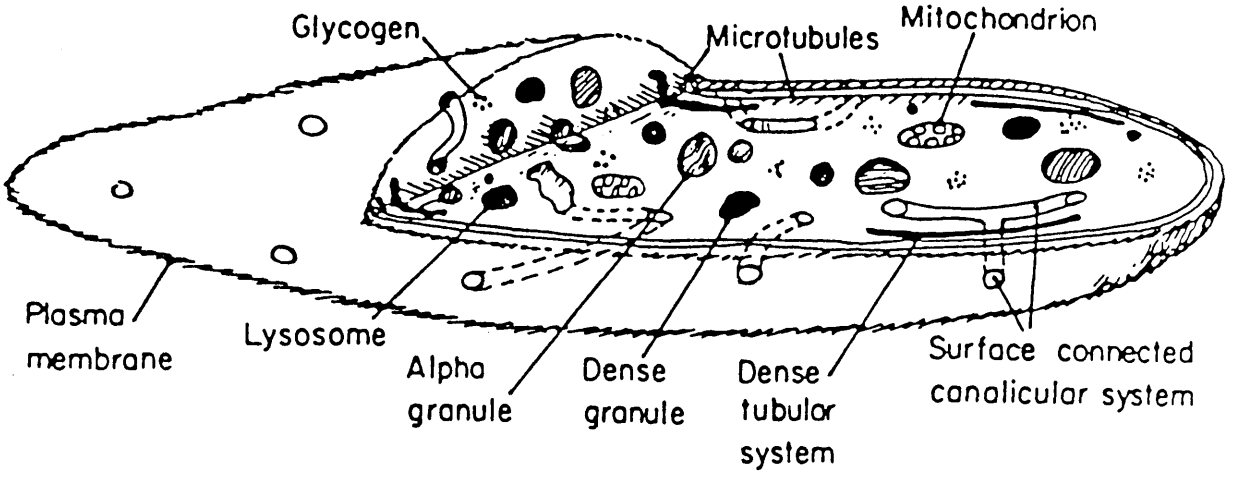

Figure 1. Illustration of the main structural features of quiescent platelets.

See text for explanation. Taken from Nichols et al. (1981).

Platelets contain a number of different granular organelles. The dense granules (dense bodies) contain high concentrations of amines, particularly 5HT, and adenine nucleotides (ADP, ATP) and bivalent cations (Ca^{2+}, Mg^{2+}). 5HT is taken up into platelets and then into dense granules by a selective active transport process which mops up free plasma 5HT (Humphrey & Toh, 1954). The dense granule contents are secreted upon platelet activation. Alpha granules and lysosomes are the other main storage organelles whose contents are secreted upon platelet activation. The alpha granules are heterogeneous in nature and contain a range of proteins including (1) platelet-factor 4 (PF4) and β-thromboglobulin (both are anti-heparin proteins), (2) low affinity PF4 (a precursor of β-thromboglobulin), (3) platelet-derived growth factor and β-transforming growth factor (which stimulate smooth muscle proliferation and collagen formation by fibroblasts), (4) fibrinectin, fibrinogen and thrombospondin (aggregation cofactors), (5) Von Willebrand Factor (possibly involved in mediation of platelet adhesion to arterial subendothelium) and (6) albumin (Kaplan, 1981). The lysosomes contain a range of enzymes including acid phosphatase and β-N-acetylglucosaminidase. Platelets also contain glycogen granules (the smallest and most abundant platelet granules) and mitochondria.

III. Platelet reactions

The main reactions which platelets undergo in response to stimulation are shape change, adhesion, aggregation, secretion of granule contents and eicosanoid synthesis and secretion.

III.1 Shape change

As previously described (Section II), the characteristic discoid shape of platelets is maintained by the orderly circular arrangement of the microtubules. The microtubule organisation is disrupted when the platelets are activated and the other elements of the platelet's structural and contractile apparatus, actin and myosin, interact leading to a dramatic and rapid change in platelet shape. This shape change is isovolemic and consists of a rounding-up of the central platelet body and the extension of long thin pseudopodia which effectively increase the platelet surface area and facilitate the platelet-platelet contact essential for aggregation (Born, 1970; Gordon, 1981). Platelet shape change can be monitored, in vitro, by measuring the transmission and/or scattering of light through plasma or washed platelet suspensions. Shape change results in an increase in optical density and hence a decrease in light transmission (Michal & Born, 1971).

III.2 Adhesion

Platelets will readily adhere to exposed vascular collagen and this can lead to aggregation and secretion of granule contents. This is the primary step in the formation of a haemostatic plug. The molecular mechanisms of platelet adhesion to the subendothelium are not fully understood. However, adhesion probably involves the interaction of platelet surface glycoprotein Ib (and possibly the glycoprotein IIb-IIIa complex) and factor VIII (von Willebrand factor) with the exposed vascular collagen (Hardisty, 1985). Platelets do not

readily adhere to other biological macromolecules but will adhere to a variety of artificial surfaces. Adhesion can be quantitated *in vitro* by a number of different techniques. In its simplest form, this can be achieved by passing a blood sample through a column of glass beads and measuring the difference in platelet count before and after passage through the column (Salzman, 1963). However, more physiologically relevant models have been developed which involve perfusing collagen-coated coverslips or endothelium-denuded blood vessels at physiological shear rates (Baumgartner & Mugli, 1976; Sakariassen et al., 1983).

III.3 Aggregation

As well as sticking to foreign surfaces, activated platelets will also stick to each other resulting in the formation of a multicellular mass or aggregate. This process is known as aggregation. Platelet aggregation is normally preceded by shape change. Adrenaline, however, is unusual in causing a qualitatively distinct change in platelet shape without pseudopodia formation. This has been described as 'mild' shape change and occurs in the absence of changes in light transmission (Gerrard *et al.*, 1981). Reversible or 'primary' aggregation can be induced by low agonist concentrations. Irreversible or 'secondary' aggregation can be induced by higher agonist concentrations and is associated with secretion of granule contents and prostanoid formation (Smith & Willis, 1971). Aggregation requires platelet-platelet collision and extracellular cofactors including Ca^{2+} and fibrinogen to form bridges between colliding platelets (Mustard *et al.*, 1978).

The platelet glycoprotein IIb-IIIa complex, which is exposed at the surface membrane upon stimulation, functions as the receptor for fibrinogen. The glycoprotein IIb-IIIa-fibrinogen complex is stabilised by thrombospondin which is released from the alpha granules (Silverstein et al., 1986). Aggregation can be monitored in plasma or washed platelet suspensions by measuring increases in light transmission through stirred samples (Born, 1962). Aggregation can be monitored in whole blood (as well as platelet-rich plasma or washed platelet suspensions) by platelet counting where numbers of single platelets are counted by an electrical impedance method before and after addition of an aggregating agent (Lumley & Humphrey, 1981).

III.4 Secretion

(a) Degranulation

Stimulated platelets may selectively release the contents of their dense granules, alpha granules and lysosomes. The dense granule contents (5HT, ADP, ATP and Ca^{2+}) can be selectively liberated by agents such as ADP or adrenaline with little concomitant secretion of alpha granule contents or lysosomal enzymes (Mills et al., 1968). Dense granule contents (with the notable exception of ATP) are pro-aggregatory and their main function is presumably, to promote the growth of a haemostatic plug. Release of the alpha granule contents and lysosomal enzymes normally requires stronger stimuli e.g. high concentrations of agonists like thrombin and collagen (Day & Holmsen, 1971). However, some of the contents of the heterogeneous alpha granule population are secreted more readily

than the dense granule contents. Secretion is associated with granule centralisation which may be mediated by actomyosin contraction. Degranulation may result from fusion of the granule membranes with the inner parts of the SCCS (White, 1973). Secretion of dense granule contents can be monitored by measuring released ATP by luciferin-luciferase luminescence (Feinman et al., 1977) or by a radio-isotopic (^{14}C- or ^{3}H-5HT) prelabelling method.

(b) Secretion of newly-synthesized platelet products.

Stimulated platelets release arachidonic acid which is rapidly converted to pro-aggregatory eicosanoids prostaglandin (PG)G_2, PGH_2 and thromboxane (Tx)A_2. Eicosanoid formation constitutes a positive feedback mechanism to stimulate further platelet aggregation, promote growth of a haemostatic plug and cause local vascular constriction (Moncada & Vane, 1979). The mechanisms and significance of prostanoid formation are discussed below (Section V.3). Platelet arachidonic acid is also metabolised by 12-lipoxygenase, especially in the presence of inhibitors of cyclooxygenase such as aspirin or indomethacin. This pathway of arachidonic acid metabolism leads to production of 12-hydroperoxy-eicosatetraenoic acid (12-HPETE) which is rapidly converted to 12-hydroxy-eicosatetraenoic acid (12-HETE). Both are biologically active (12-HPETE > 12-HETE) as (1) chemotactic and chemoattractant agents; (2) stimulators of neutrophil degranulation; (3) probable mediators of anaphylactic contraction of lung smooth muscle and (4) inhibitors of platelet activation (Vargaftig et al., 1981). Eicosanoid formation and release can be monitored by a radio-isotopic (^{3}H- or ^{14}C-arachidonic acid) prelabelling technique; by bioassay of the

active eicosanoids or by radioimmunoassay of the inactive breakdown products (e.g. TxB_2 which is formed from TxA_2).

Activated platelets (as well as other inflammatory cells) may also synthesize and release platelet-activating factor (PAF: acetyl glyceryl ether phosphoryl choline). Vargaftig et al. (1981) proposed that PAF could be formed by (1) deacetylation of membrane phospholipids, including analogues of phosphatidyl choline with an ether linkage at glycerol-sn-1 position, by a phospholipase A_2-like enzyme to form lyso-PAF followed by (2) acetylation, involving a hypothetical acetylating enzyme, at glycerol-sn-2. PAF has a wide range of biological actions, in addition to platelet activation, and may be an important mediator of inflammation (reviewed by Vargaftig et al., 1981).

III.5 Platelet energy metabolism

The energy required for the various platelet reactions is derived from glycolysis in the cytosol (utilising stored glycogen or extracellular glucose) and from oxidative phosphorylation in the mitochondria. The relative abundance of platelet glycogen granules, compared to mitochondria, is consistent with the platelet's metabolic energy being derived mainly by glycolysis rather than by oxidative phosphorylation (Gordon, 1981).

III.6 Synergism and desensitization

Platelets contain receptors for, and can be activated by, a number of agents which are normally present in the blood or which can be synthesized or secreted by the platelets themselves. When added individually to platelets, in vitro, the concentrations

of agonists such as adrenaline, ADP, PAF and vasopressin required for stimulation of platelet aggregation markedly exceed those concentrations normally present in the blood (Scrutton & Wallis, 1981). Exposure of platelets, *in vitro*, to two or more agonists can result in the synergistic stimulation of aggregation and dense granule secretion (i.e. produce a response which is significantly greater than the sum of the individual responses). Such synergistic responses have been observed when platelet aggregation is monitored turbidometrically (O'Brien, 1964; Ardlie *et al.*, 1966; Mills & Roberts, 1967; Nakanishi *et al.*, 1971; Michal & Motamed, 1976; Grant & Scrutton, 1980a; Vargaftig *et al.*, 1982). Synergistic induction of thrombocytopaenia has also been observed *in vivo* (Markwardt *et al.*, 1977). This phenomenon may be of major importance in haemostasis and in the pathogenesis of occlusive vascular disease since it may permit significant action by agents which do not obtain the physiological concentrations required to have any effect alone. Although TxA_2 formation and release of granule constituents are not prerequisite for synergism to occur (Rao *et al.*, 1980), the molecular mechanisms underlying this effect are largely unknown. It seems likely that positive agonist interactions will be mediated at the level of intracellular second messenger systems (Scrutton & Wallis, 1981). The biochemical basis of synergism has been investigated using tissues such as the parotid gland and pancreas. In the pancreas, agonist-induced enzyme release is a consequence of increased intracellular concentrations of cyclic AMP or free calcium ions. Synergism is only observed using combinations

of agonists that act through different intracellular messengers, whereas desensitization is observed with agonists that share the same intracellular messengers (Gardner & Jensen, 1980). Whether this general rule can be applied to other cellular systems such as the platelet remains to be determined.

The term desensitization describes a loss in cellular activity subsequent to the initial action of an agonist. This phenomenon occurs in a wide variety of cellular systems and is variously described as fade, tachyphylaxis, tolerance, refractoriness or subsensitivity. In specific, or homologous, desensitization pre-treatment with an agonist will inhibit responsiveness to the subsequent addition of agonists which act through the same but not different receptors. Non-specific or heterologous desensitization is associated with a general decrease in cellular responsiveness, irrespective of the receptors involved. Homologous desensitization is generally attributed to events at the specific receptor whilst heterologous desensitization is mediated via an effect(s) on the transduction processes which are common to different receptor types (see Section V).

There are a number of different potential mechanisms of desensitization. Acute desensitization of skeletal muscle nicotinic receptors is associated with rapid and transient changes in agonist-receptor binding affinity. Chronic desensitization develops more slowly, is less rapidly reversible and is accompanied by receptor loss (down-regulation) and reduction in receptor sensitivity (Heidman & Changeux, 1978). Desensitization of hormone sensitive adenylate cyclase has

been well characterised. In avian erythrocyte membranes, desensitization of β-adrenoceptor-mediated activation of adenylate cyclase is agonist-dependent and is prevented or reversed by antagonists (Lefkowitz & Williams, 1978). . In human astrocytoma cells, desensitization is apparently mediated by cyclic AMP-dependent phosphorylation reactions (Su et al., 1976), and in rat astrocytoma cells cyclic AMP-dependent desensitization is dependent on protein synthesis, presumably reflecting the generation of an endogenous inhibitor (Nickols & Brooker, 1979). The process of heterologous desensitization is dependent upon cellular integrity and does not occur in membrane preparations.

The smooth muscle of guinea pig ileum responds to a range of compounds including acetylcholine, histamine and substance P. These are all coupled to stimulation of calcium influx from the extracellular space. In this system, substance P will induce homologous desensitization whilst both acetylcholine and histamine induce heterologous desensitization (Triggle, 1980). Here, homologous desensitization may reflect changes at the receptor level and heterologous desensitization is probably caused by membrane hyperpolarisation resulting from the electrogenic pumping of sodium ions that enter the smooth muscle cells during stimulus-induced depolarisation (Triggle, 1980).

Desensitization is also known to occur in studies of platelets in vitro. Homologous desensitization can be demonstrate using a range of agonists including ADP, thrombin and vasopressin: addition of a subthreshold concentration of an agonist will

inhibit responsiveness to the subsequent addition of the same agonist or agonists that act at the same but not other receptors (Schuman et al., 1978; Huang & Detwiler, 1986; Pollock & MacIntyre, 1986). Although the molecular mechanisms of desensitization in platelets are not fully understood, the changes occurring in homologous desensitization are probably located at the receptor level rather than the common transduction processes of phosphoinositide hydrolysis and elevation of Ca^{2+}_{i} (MacIntyre & Pollock, 1986). Recent evidence indicates a possible role for protein kinase C (see Section V) in the induction of desensitization in a number of different cellular systems including platelets (Sibley et al., 1984; Drummond & MacIntyre, 1985).

IV. Physiological and pathological roles of platelets

The main function of platelets, in vivo, is to adhere to damaged blood vessel walls and initiate the haemostatic process necessary for the repair of vascular injury. Platelets do this firstly by adhering to the subendothelial collagen and secreting pro-aggregatory compounds such as ADP, 5HT and arachidonic acid metabolites in order to recruit more platelets into the haemostatic plug and secondly, by promoting efficient coagulation near the site of injury. Platelets promote coagulation in a number of ways: (1) by exposing platelet factor 3 (a procoagulant phospholipid complex) on their surface membrane in response to stimulation by collagen; (2) By carrying coagulation factor XI on their membranes which becomes activated when the platelets are in contact with collagen and (3) by secreting procoagulant proteins from their alpha granules

(e.g. fibrinogen, platelet factor 4 and β-thromboglobulin). Coagulation results from the conversion of prothrombin to thrombin which is itself a powerful platelet stimulant and which catalyses the conversion of fibrinogen to strands of fibrin which consolidate the haemostatic plug. Platelets may also promote wound healing by secreting mitogenic proteins including PDGF and β-transforming growth factor which can stimulate smooth muscle proliferation (Gordon, 1981). Evidence in support of a role for platelets in haemostasis comes from the observation that deficiencies or defects in circulating platelets are associated with spontaneous haemorrhages from small vessels (e.g. see Tschopp et al., 1974; Gordon & Milner, 1976).

Platelets may also be involved in the maintenance of endothelial integrity. This is supported by the finding of increased vascular permeability in thrombocytopaenic animals (Johnson, 1971). Platelets are capable of pinocytosis and exocytosis and may play an important role in scavenging particles from the blood (Van Aken & Vreeken, 1969). There is some evidence that platelets could be involved in the inflammatory process (1) directly, by secreting agents which increase vascular permeability (e.g. 5HT) and (2) indirectly by stimulating leucocyte chemotaxis (via products of 12-lipoxygenase activity) and mast cell degranulation (Gordon & Milner, 1976).

The main pathological role of platelets is in the development of occlusive vascular disease by promoting thrombosis and atherosclerosis. Thrombosis is the pathological perversion of

haemostasis and is characterised by thrombus formation in the presence or absence of vascular damage. Arterial thrombogenesis may be initiated by vascular trauma or by platelets interacting with damaged endothelial cells or by 'hyper-reactive' platelets forming aggregates without any vascular contact. The nature of the platelet or endothelial cell changes which promote thrombogenesis in the absence of gross vascular damage are largely unknown. Thrombosis can prove fatal when it results in occlusion of the cardiac or cerebral vasculature. Atherosclerosis is characterised by localised narrowing of the vascular lumen caused by formation of plaques containing fibrous tissue and lipid which produces focal thickening of the arterial intima. Although the contribution of platelets to atherogenesis is not fully established, they may participate in a number of ways: (1) by releasing agents which damage arterial endothelial cells (Mustard et al., 1977); (2) by releasing factors which stimulate smooth muscle proliferation in the arterial wall (Ross & Glomset, 1973) and (3) through their involvement in the formation of persistent mural thrombi which are subsequently organised into intimal thickenings (Gordon & Milner, 1976; Gordon, 1981). Platelets may also be involved in the pathological aspect of inflammation by releasing (a) a variety of inflammatory stimuli, e.g. PAF, eicosanoids, 5 HT; (b) lysosomal enzymes capable of degrading connective tissue and possibly (c) proteins that can increase vascular permeability (Gordon & Milner, 1976).

V. Agonist-receptor-controlled transduction processes

Platelet responsiveness can be modulated by a number of different compounds which act to promote (stimulatory agonists) or retard (inhibitory agonists) platelet activation. These agonists are diverse in nature and act upon specific recognition sites or 'receptors'. Some of the platelet agonists which are thought to be of patho-physiological importance in the control of platelet reactivity and the receptor sub-types with which they combine to mediate their effects (where known) are listed in Table 1. Platelet receptors for agonist are generally assumed to be located on the plasma membrane although intracellular receptors may exist for lipophilic compounds such as TxA_2, the prostaglandins or PAF. Hence, transduction mechanisms must exist to allow the translation of external signals (i.e. agonist-receptor interaction) into changes in cellular responsiveness. This is normally achieved by the generation of internal signals which are carried by 'second messengers'. The second messengers can then either remain in the membrane or diffuse throughout the cell and combine with specific intracellular acceptors to modulate the activity or rates of the key biochemical reactions which control the cellular response (Figure 2; Berridge, 1981).

Since platelets are electrically non-excitable (MacIntyre & Rink, 1982; Doyle & Ruegg, 1985), stimulus-induced changes in platelet reactivity must be mediated by chemical or electroneutral ionic processes. Indeed, the second messengers which thus far have been identified as important in the control of platelet reactivity are cyclic AMP, 1,2-diacylglycerol (DG),

TABLE 1. Some common platelet agonists and receptors.

Agonist	Receptor	Reference	Response
Thrombin	*N.C.	Detwiler & McGowan, 1985	Stimulation
Collagen	*N.C.	—	
ADP	P_{2T}	Gordon, 1986	
TxA_2/PG endoperoxide	TP	Kennedy et al., 1983	
PAF	Paf_1	Lambrecht & Parnham, 1986	
Vasopressin	V_1	Pollock & MacIntyre, 1986	
5HT	$5HT_2(S_2)$	de Chaffoyde Courcelles et al., 1985	
Adrenaline	α_2	Kerry & Scrutton, 1985	
PGI_2/PGE_1	IP	Kennedy et al., 1983	Inhibition
PGD_2	DP	Kennedy et al., 1983	
Adenosine	A_2	Daly, 1982	
Adrenaline	β	Jakobs et al., 1978	

*N.C. = receptor subtypes not classified.

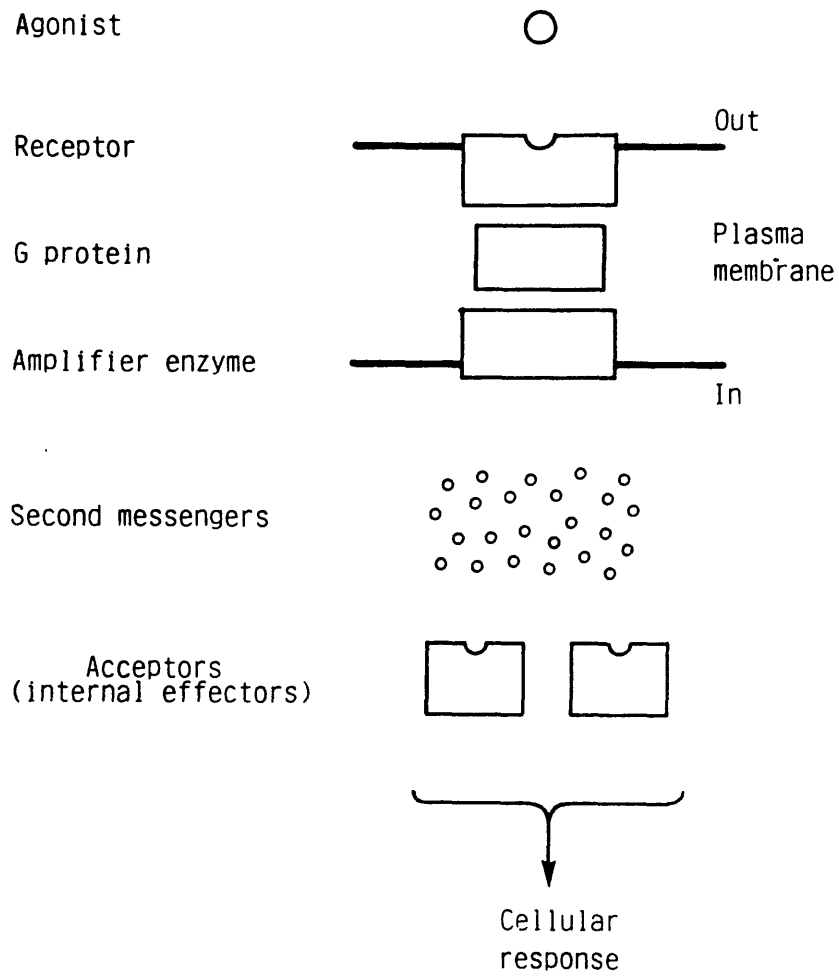

Figure 2. General mechanism of stimulus-response coupling.

See text for explanation.

inositol 1,4,5-trisphosphate ($Ins(1,4,5)P_3$) and $[Ca^{2+}]_i$. Inhibition of platelet responsiveness is associated with agonist-induced elevation of the intracellular concentration of cyclic AMP (Haslam et al., 1978) whilst stimulation is associated with agonist-induced elevations in the intracellular concentrations of DG, $Ins(1,4,5)P_3$ and $[Ca^{2+}]_i$ (Kaibuchi et al., 1983; Streb et al., 1983; O'Rourke et al., 1985; Rink et al., 1982). The two second messenger pathways apparently have much in common (Fig. 2). In both, the receptor molecule on the outer surface of the cell transmits information across the plasma membrane via a family of 'G' proteins: guanine nucleotide-binding regulatory proteins which become active upon binding of guanosine triphosphate (GTP). The G proteins activate an 'amplifier' enzyme located on the inner face of the membrane and which then converts precursor molecules into second messengers. In the inhibitory second messenger pathway the enzyme adenylate cyclase converts Mg^{2+}.ATP into cyclic AMP whilst in the stimulatory pathway, the enzyme phospholipase C converts the membrane inositol-containing phospholipids (phosphatidyl inositol 4,5-bisphosphate: PIP_2 and possibly also phosphatidyl inositol: Ptd Ins and phosphatidyl inositol 4-phosphate: PIP) into DG and the corresponding inositol phosphates including $Ins(1,4,5)P_3$. The final step in both pathways is also similar: the second messengers interact with their acceptor proteins to induce a conformational change in their three dimensional structure. The acceptors for cyclic AMP, DG, and in some cases Ca^{2+}, are components of distinct protein kinase enzymes which, upon activation, can phosphorylate

various platelet proteins to induce functional changes (Krebs, 1984; Berridge, 1985). However, Ca^{2+} can also directly modulate the activity of some enzymes in the absence of increased phosphorylation. Platelet responsiveness is ultimately controlled by the interactions of the two different second messenger pathways which effectively form a bidirectional control system similar to that defined by Berridge (1975).

In addition to the second messenger pathways so far discussed, there may also be a role for cyclic GMP in the control of platelet function. Agents such as sodium nitroprusside, which elevate intracellular cyclic GMP levels inhibit platelet function by a method which is poorly understood (Haslam et al., 1980). Cyclic GMP levels have been shown to rise in response to the addition of certain platelet stimulatory agonists. This occurs after a relatively long lag phase (Haslam et al., 1980). In some other cell types, guanylate cyclase, the enzyme that makes cyclic GMP from GTP, is not connected to a receptor and cyclic GMP formation often occurs after the activation of phosphoinositide hydrolysis (Berridge, 1985). Since, in platelets, cyclic GMP inhibits agonist-induced elevation of $\left[Ca^{2+}\right]_i$, it is possible that cyclic GMP could act as a negative feed-back regulator to limit platelet responsiveness (MacIntyre et al., 1985a). Changes in intracellular pH have also been shown to correlate with a number of cellular processes, suggesting a possible second messenger role for H^+. It has been suggested that thrombin-induced platelet activation correlates with an increase in cytosolic pH (Horne et al., 1981). However, this finding is disputed (Rotman & Heldman, 1982) and the lack of effect of other

agonists on intracellular pH indicates that this may not be an important regulatory mechanism in platelets (Huang & Detwiler, 1986). The receptor-controlled second messenger systems are discussed in more detail below.

V.1. Cyclic AMP and platelet function

One of the first indications that cyclic AMP might be involved in the regulation of platelet function was the observation by Marcus and Zucker (1965) that addition of cyclic AMP to platelets resulted in inhibition of platelet aggregation. This was supported by the observation that phosphodiesterase inhibitors blocked ADP-induced aggregation (Ardlie et al., 1967). Since then, it has become clear that many of the agents which inhibit platelet activation produce their effect through increases in cyclic AMP levels (Haslam et al., 1978). Besides inhibiting the functional platelet responses (Hawiger et al., 1980; Feinstein et al., 1981) initiated after exposure to stimulatory agonists, the inhibitory effects of cyclic AMP are also evident on the transduction processes that link receptor occupancy to cellular activation (Kaser-Glanzmann et al., 1977; Fox et al., 1979; Feinstein et al., 1983; Sano et al., 1983; Lapetina, 1984). Platelet receptors for some agonists can be coupled to inhibition of adenylate cyclase activity (Aktories & Jakobs, 1985). Whether agonist-induced platelet activation could result from a decrease in the basal level of cyclic AMP, perhaps in some specific pool, is controversial (Salzman, 1972; Haslam et al., 1978).

Since the initial discovery that cyclic AMP acts as an intracellular second messenger (Sutherland & Rall, 1960) intensive research efforts have been devoted to unravelling the detailed control mechanisms which regulate the generation and subsequent actions of this molecule. Studies in various cell types have clearly indicated that the membrane-bound, hormone-sensitive cyclic AMP generating system is composed of at least three distinct subunits. These include the receptors which discriminate the specificity of the external signals; the G proteins and adenylate cyclase which is the catalytic subunit of the complex (Schramm et al., 1977; Rodbell, 1980; Schramm & Selinger, 1984; Aktories & Jakobs, 1985). This is illustrated schematically in Figure 3.

V.1.(1) Receptors

Some of the agonists and receptors coupled to stimulation or inhibition of adenylate cyclase in platelets are listed in Table 2. Receptors for PGI_2 have been identified by radioligand-binding studies: two classes of binding site have been identified: a low affinity, high capacity site (Kd = 1.6 µM; 4000 sites per cell) and a high affinity, low capacity site (Kd = 10 nM, 50 sites per cell) which apparently corresponds to the functional PGI_2 receptor (Siegl, 1982). PGE_1 and 6ketoPGE$_1$ also are agonists at the PGI_2 receptor, which has been termed an IP receptor (Kennedy et al., 1983). PGD_2 acts via distinct so-called DP receptors. Radioligand-binding studies reveal a single class of PGD_2 binding sites: Kd = 50-400 nM, 200-800 sites per cell (Siegl et al., 1979). Platelet receptors for adenosine which are coupled to stimulation of adenylate cyclase have been identified as conforming to

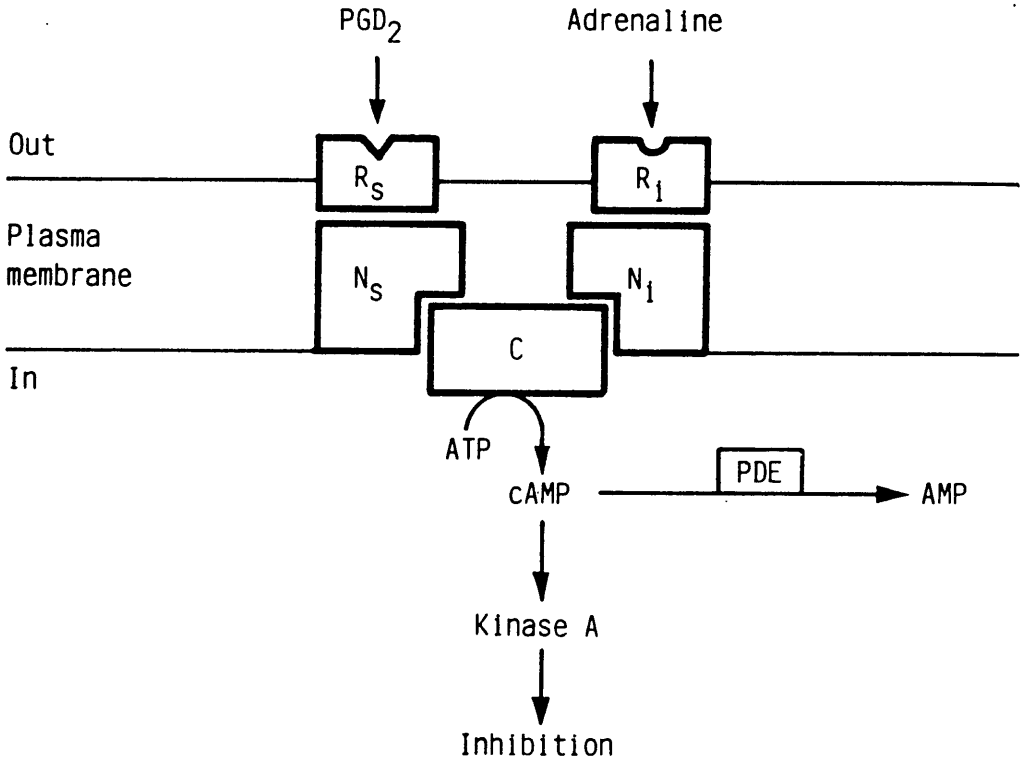

Figure 3. Schematic representation of receptor-mediated control of cyclic AMP metabolism.

See text for explanation.

TABLE 2. Agonists and receptors which influence adenylate cyclase activity in intact platelets.

Agonists (Receptors)	G Proteins
PGI_2 (IP) PGE_1 (IP) PGD_2 (DP) Adenosine (A_2) Catecholamines (β_2)	N_s
Catecholamines (α_2) ADP (P_{2T})	N_i

the A_2 subtype on the basis of structure-activity relationships (Daly, 1985). Radioligand binding analyses reveal a single class of adenosine binding sites with Kd 12 nM (Ukena et al., 1986). The presence of β-adrenoceptors on human platelets is indicated by the ability of isoprenaline and adrenaline to stimulate adenylate cyclase activity in the presence of an α-adrenoceptor blocking agent (Abdulla, 1969; Jakobs et al., 1978). Radioligand binding analyses reveal that the platelet β-adrenoceptors are of the β_2-subtype and are present in very small numbers: around 1/10 to 1/20 of the platelet α_2-adrenoceptors (Kerry & Scrutton, 1983; Barnett et al., 1985).

The predominant platelet receptor for adrenaline has been characterised as belonging to the α_2-subtype by pharmacological analyses using a range of agonists and antagonists (Hsu et al., 1979; Grant & Scrutton, 1980b; Barnett et al., 1985). The α_2-adrenoceptor is coupled to inhibition of adenylate cyclase in intact platelets (Aktories & Jakobs, 1985). Radioligand-binding studies using ^3H -yohimbine suggest that there is a single class of binding sites: Kd = 3nM and 270 sites per platelet (Kerry & Scrutton, 1985). Platelet receptors for ADP are also coupled to inhibition of adenylate cyclase in intact platelets (Haslam & Rosson, 1975). Radioligand-binding studies using a stable analogue of ADP reveal a single class of binding sites for ADP on human platelets: Kd = 15 nM and 400-1200 sites per platelet (Macfarlane et al., 1983). Pharmacological analyses using a range of compounds indicate that the receptor for ADP on human platelets is unique and it has thus been designated a P_{2T}-purinoreceptor (Cusack et al., 1985; Gordon, 1986).

Receptors for PAF, vasopressin and thrombin can also be coupled to inhibition of adenylate cyclase in platelet lysates, but not in intact cells (Haslam & Vanderwel, 1982; Vanderwel et al., 1983; Aktories & Jakobs, 1984). Kloprogge & Akkerman (1983) have identified a single class of PAF binding sites (Kd = 17 nM; 280 sites per cell) whilst Tuffin et al. (1985) have identified two distinct PAF binding sites. Platelet receptors for PAF have been termed Paf_1 and are distinct from the Paf_2 receptor subtype present on macrophages (Lambrecht & Parnham, 1986). Platelet receptors for vasopressin belong to the V_1 receptor subtype. Radioligand-binding analyses reveal a single class of binding sites Kd ranging from 1.3 - 24 nM (depending upon the presence or absence of divalent cations) and 90-150 sites per cell (Pletscher et al., 1985; Vittet et al., 1986). The serine protease thrombin, is the most potent and efficacious platelet agonist. Two classes of thrombin binding site have been demonstrated: a high affinity low capacity site (Kd = 10 nm, 500 sites per cell) and a lower affinity high capacity site (Kd = 100 nM, 50,000 sites per cell) (Detwiler & McGowan, 1985). Whether thrombin-induced platelet activation is a consequence of proteolytic hydrolysis of a particular substrate or of a classical agonist-receptor interaction is not known (MacIntyre et al., 1986).

There is some dispute over whether receptors for TxA_2 are coupled to inhibition of adenylate cyclase in intact platelets. Miller et al. (1977) reported that TxA_2 inhibited PGE_1-stimulated cyclic AMP accumulation whilst Best et al. (1979) found no effect of the stable TxA_2 analogues U44069 and U46619

on PGE_1-induced cyclic AMP formation. Using radiolabelled U44069 as ligand, two high affinity binding sites have been identified, one of which (Kd = 70 nM; 2000 sites per cell) has the pharmacological characteristics of the receptor mediating platelet activation (Armstrong et al., 1983). Platelet receptors for TxA_2 have been termed TP receptors (Kennedy et al., 1983) but may differ from TxA_2 receptors situated elsewhere e.g. vascular smooth muscle.

V.1.(2) G Proteins

The stimulatory G protein, N_s, was first identified with GTP-photoaffinity analogues as a heat-stable 42,000 Mr protein in detergent extracts of avian erythrocyte membranes (Pfeuffer, 1977; 1979). The binding of an agonist to the stimulatory receptor, R_s, induces a conformational change which is transmitted through the plasma membrane to N_s, making it more susceptible to GTP binding. The binding of GTP to N_s constitutes an 'on-reaction'. That is, a conformational change occurs enabling N_s to activate adenylate cyclase resulting in increased formation of cyclic AMP. GTP bound to N_s is then hydrolysed to form GDP resulting in loss of activity of N_s. This 'off-reaction' is catalysed by the GTPase activity of N_s (Rodbell, 1980). The conformational changes in N_s resulting from GTP binding are reflected in changes in the agonist binding affinity of R_s. GTP-N_s binding generally results in reduction of agonist high affinity binding at R_s (Jakobs et al., 1986). Cholera toxin has been shown to ADP-ribosylate N_s in the presence of a protein cofactor termed ADP-ribosylation factor: ARF (Kahn

& Gilman, 1984). This leads to inhibition of GTPase activity and prevention of the off-reaction, effectively resulting in prolonged stimulation of cyclic AMP production. This has been observed in a variety of cell types, even in the absence of hormones (Finkelstein, 1973; Cassel & Selinger, 1977; Cassel & Pfeuffer, 1978). This property of cholera toxin has made it a valuable tool in probing the role of N_s. However, this toxin does not increase adenylate cyclase activity when added to intact platelets. Human platelets are apparently defective in ganglioside receptor(s) and/or other factors required for processing and activating cholera toxin (Hughes & Insel, 1983; Sekura et al., 1985).

The existence of an inhibitory G protein, N_i, was first indicated by the ability of GTP to inhibit adenylate cyclase in rat adipocyte membranes (Creyer et al., 1969). As with the stimulatory system, the binding of agonists to inhibitory receptors, R_i, results in association of R_i with N_i and the binding of GTP to N_i. This leads to a conformational change in N_i which allows it to inhibit adenylate cyclase. Subsequent hydrolysis of GTP to GDP results in loss of inhibitory activity of N_i (Rodbell, 1980). GTP-binding to N_i results in modification of the high affinity binding of agonists to R_i (Grandt et al., 1982). Pertussis toxin (islet-activating protein) has been shown to ADP-ribosylate N_i resulting in inhibition of formation of the activated N_i-GTP species thereby attenuating agonist-R_i-dependent control of adenylate cyclase activity (Birnbaumer et al., 1985). This property of pertussis toxin has proven very useful in probing the role of N_i in stimulus-response coupling. Pertussis toxin has no effect on cyclic AMP metabolism

in intact platelets since they lack the glycoprotein(s) or ganglioside(s) required for binding and uptake of the toxin into the cytosol (Ui, 1984; Sekura et al., 1985). N_i-mediated inhibition of adenylate cyclase can also be attenuated by proteolytic enzymes such as trypsin and the sperm protease(s) known as ninhibin (Jakobs et al., 1984).

N_s and N_i are structurally similar heterotrimers consisting of a larger α-subunit (41 - 45,000 M_r) and two smaller subunits, β : 35,000 M_r and γ : 10,000 M_r (Gilman, 1984). The β- and γ-subunits of N_s and N_i are functionally indistinguishable. These two subunits are closely associated as a βγ- complex and are assumed to act as inhibitors of the α-subunits (Smigel et al., 1984). It has been proposed that, upon agonist-receptor interaction, the G proteins dissociate into free α-subunits and βγ-complexes (Gilman, 1984). It is the α-subunits which are substrates for ADP-ribosylation in the presence of cholera toxin (α_s) or pertussin toxin (α_i) (Ui, 1984). G protein functioning can also be modulated by stable (non-hydrolysable) analogues of GTP (GppNHp, GTPγS) or GDP (GDPβS) resulting in persistent activation or inhibition, respectively (Jakobs & Aktories, 1983).

V.1 (3) Adenylate cyclase

Compared to the detailed knowledge of the structure and function of agonist receptors and G proteins, relatively little is known of the structure and functioning of the adenylate cyclase subunit itself. It has recently been purified from rabbit myocardium and identified as a glycoprotein (M_r 150,000)

which traverses the plasma membrane (Pfeuffer et al., 1985). Activity of the adenylate cyclase moiety can be modulated independently of receptors or G proteins by a number of agents. The diterpene, forskolin, stimulates adenylate cyclase from all cell types so far examined. This effect is probably mediated via interaction with a site or sites on the catalytic subunit or closely related protein (Seamon & Daly, 1981). In addition, forskolin also markedly potentiates R_s-N_s-stimulated adenylate cyclase activity, particularly in intact cells (Daly, 1984). The apparently incongruous finding that low concentrations of forskolin can inhibit adenylate cyclase activity of platelet membrane preparations has recently been reported (Watanabe & Jakobs, 1986). Adenosine and some of its analogues can inhibit adenylate cyclase activity via an action at the so-called 'P-site' which is probably associated with the catalytic subunit (Londos & Wolf, 1977; Florio & Ross, 1983). The physiological role of this P-site action of adenosine analogues is uncertain but it may represent an endogenous feedback mechanism to limit agonist-induced stimulation of adenylate cyclase (Jakobs et al., 1986).

V.1.(4) Cyclic AMP phosphodiesterase

The other important enzyme in the control of cyclic AMP metabolism is cyclic 3',5'-nucleotide phosphodiesterase which catalyses the inactivation of cyclic AMP by conversion to 5'-AMP. This enzyme was originally studied by Sutherland and colleagues (Butcher & Sutherland, 1962) and it is now clear that multiple forms of phosphodiesterase exist. The different

isozymes have been classified according to their substrate specificity: cyclic nucleotide phosphodiesterase (hydrolyses both cyclic AMP and cyclic GMP), cyclic AMP phosphodiesterase and cyclic GMP phosphodiesterase. Subclassifications within these groups are based upon the regulatory and kinetic properties of the isozymes. Thus, cyclic AMP phosphodiesterase may be Ca^{2+}/calmodulin-stimulated, cyclic GMP- or cyclic AMP-stimulated and may have low or high 'Km' (Beavo et al., 1982). Pichard & Cheung (1976) reported three interconvertible forms of cyclic nucleotide phosphodiesterase in platelet soluble fractions. The rate of cyclic AMP metabolism was stimulated by cyclic AMP. Coquil et al. (1980) reported the presence of a cyclic GMP-stimulated soluble cyclic nucleotide phosphodiesterase in platelets.

Elevations in platelet cyclic AMP content in response to inhibitory agonists like PGI_2 or PGD_2 are transient. The delayed decline in cyclic AMP levels correlates with increased cyclic AMP phosphodiesterase activity. This activation of phosphodiesterase activity is an indirect effect which may occur as a consequence of elevated cyclic AMP levels and could represent a homeostatic mechanism for the regulation of platelet cyclic AMP metabolism (Pichard & Cheung, 1976; Alvarez et al., 1981). Cyclic nucleotide phosphodiesterase activity can be inhibited by 1-isobutyl 3-methyl xanthine (Marquis et al., 1969). This results in elevation of intracellular cyclic AMP concentration and marked potentiation of agonist-induced elevations in cyclic AMP levels (Alvarez et al., 1981).

V.1. (5) Mechanism of action of cyclic AMP

Evidence from different cell types indicates that the many actions of cyclic AMP are mediated via activation of a protein kinase enzyme called cyclic AMP-dependent protein kinase or kinase A (Flockart & Corbin, 1982; Krebs, 1984; Berridge, 1985). This enzyme has two principal classes of isozymes labelled type I and type II on the basis of their elution from DEAE-cellulose (Corbin et al., 1975). The ratio of these different isozymes and their subcellular distribution varies in different tissues (Corbin et al., 1975). The enzymes are tetramers consisting of two regulatory (R) and two catalytic (C) subunits. The catalytic subunits are identical in both isozymes and the molecular weight is around 40,000 M_r. The regulatory subunits have molecular weights of around 49,000 M_r: type I and 54-56,000 M_r: type II (Flockhart & Corbin, 1982). The binding of cyclic AMP to the regulatory subunits induces a conformational change causing a decrease in the affinity of the regulatory dimer for the catalytic subunits. This results in the physical dissociation of the catalytic and regulatory subunits according to the following equation (Corbin, 1983):-

$$R_2C_2 + 4\text{cyclic AMP} \rightleftharpoons R_2\cdot(\text{cyclic AMP})_4 + 2C$$

When the catalytic subunits are bound to R, they are inactive and the addition of cyclic AMP results in the liberation of free active C which can phosphorylate a number of different proteins (Krebs, 1984). Each R subunit monomer contains two different cyclic AMP binding sites and this allows intra-

chain cooperativity for cyclic AMP binding (Corbin et al., 1982; Beeb et al., 1984). Thus, small increments in intracellular cyclic AMP concentration can result in marked increases in cyclic AMP bound to R and significant enhancement of cyclic AMP-dependent protein kinase activity. For this reason, measuring cyclic AMP-dependent protein kinase activity ratio (i.e. the fraction of this enzyme which is in the active form) often gives a very sensitive index of small changes in cellular cyclic AMP content (Corbin, 1983).

The importance of cyclic AMP-dependent phosphorylation in the control of platelet reactivity has been suggested by a number of findings. Inhibition of platelet activation by agents which elevate cyclic AMP content is associated with enhanced phosphorylation of several platelet proteins with apparent M_r of 22,000: 24,000; 50,000 and 240,000 (Haslam et al., 1980; Huang & Detwiler, 1986). Addition of PGE_1 to platelets results in increased phosphorylation of polypeptides which may be involved in the stimulation of active transport of Ca^{2+} (Haslam et al., 1979; Kaser-Glanzmann et al., 1979). In addition, purified myosin light-chain kinase is inhibited after phosphorylation catalysed by cyclic AMP-dependent protein kinase (Hathaway et al., 1981). Both types I and II cyclic AMP-dependent protein kinase are present in platelets. Type I is mostly membrane-bound while type II is located in the cytosol (Salama & Haslam, 1984). Hence, circumstantial evidence indicates that inhibition of platelet activation by cyclic AMP is mediated via cyclic AMP-dependent protein kinase(s).

Evidence from a number of different cell types indicates that cyclic AMP often acts primarily to modulate the activity of another second messenger pathway (Berridge, 1985). In agreement with this general rule, cyclic AMP acts in platelets to modify the rate of production and inactivation of the stimulatory second messenger molecules Ca^{2+} and DAG (Kaser-Glanzmann et al., 1977; Feinstein et al., 1983; Lapetina, 1984). Moreover, besides influencing the concentration of stimulatory second messengers, cyclic AMP may also antagonize their effects to prevent or reverse agonist-induced platelet activation (Hathaway et al., 1981).

V.2. Calcium, inositol phospholipid metabolism and platelet function

V.2.(1) Role of calcium in stimulus-response coupling

The importance of calcium in cellular physiology was first indicated over 100 years ago when Ringer reported that frog muscle contraction only occurred when calcium was present in the bathing medium (Ringer, 1883). Since then it has become clear that intracellular free Ca^{2+} regulates not only contraction but also many other cellular processes. A role for Ca^{2+} in the control of platelet reactivity was first suggested by the observation that Ca^{2+} ions were required for thrombin-induced secretion at 15°C but not at 37°C (Grette, 1962). Studies using the ionophore A23187 provided the best early evidence that Ca^{2+} is involved in mediating the responses of platelets to stimulation; i.e. shape change, aggregation, degranulation, glycogenolysis and arachidonic acid liberation

(Feinman & Detwiler, 1974; White et al., 1974; Massini & Luscher, 1974). The extents of dense granule release and prostanoid biosynthesis in response to A23187 are at least equivalent to those induced by thrombin (Feinman & Detwiler, 1974). Interestingly, A23187 was shown to be ineffective as an activator of phospholinositide metabolism (Rittenhouse-Simmons, 1981).

Until recently, direct measurement of platelet cytosolic free calcium ion concentration ($[Ca^{2+}]_i$) has been difficult since platelets are too small to allow introduction of electrodes or microinjection of indicators. Hence several indirect approaches have been used with some success. Chlortetracycline has been used in platelets as a probe of membrane-bound Ca^{2+} (Caswell, 1972). This technique has been used to demonstrate a decrease in membrane-associated Ca^{2+}, measured as a decrease in fluorescence, which preceded secretion in response to thrombin or trypsin (Feinstein, 1980). This was the first evidence of an agonist-induced shift in intracellular Ca^{2+} which preceded the response. Agonist-induced modulation of $^{45}Ca^{2+}$ association with platelets has also been monitored (Owen et al., 1980). $^{45}Ca^{2+}$ binds to the outer surface of the platelet plasma membrane and can also be taken up into two intracellular pools: a rapidly exchanging pool located in the cytosol and a more slowly exchanging pool which probably represents the DTS (Brass, 1984). It has been reported that adrenaline, but not ADP, stimulates increased $^{45}Ca^{2+}$ association with platelets and this apparently represents an increase in

both $^{45}Ca^{2+}$-binding and $^{45}Ca^{2+}$ influx into the platelets (Owen et al., 1980). The role of $[Ca^{2+}]_i$ in the control of platelet function has also been investigated using platelets made permeable to Ca^{2+} and other small molecules, by high voltage electrical discharge. Thus, $[Ca^{2+}]_i$ could be manipulated by alteration of the extracellular milieu thereby allowing equilibration of $[Ca^{2+}]_i$ with the imposed $[Ca^{2+}]_o$ (Knight & Scrutton, 1980).

Recently, a technique has been developed which allows direct measurement of $[Ca^{2+}]_i$ in small intact cells including platelets (Tsien, 1980; Tsien et al., 1982a,b). $[Ca^{2+}]_i$ is reported by the fluorescence of a Ca^{2+}-indicating dye trapped in the cytosol. The dye, called Quin2, is a calcium chelator which is relatively indifferent to Mg^{2+} and H^+ and undergoes fluorescence changes in response to calcium binding. Although Quin2 is a polycarboxylate anion which seems not to associate with or cross membranes significantly, it can be incorporated into intact cells if the carboxylate groups are suitably esterified. The resulting derivative, Quin2-acetoxymethyl ester (Quin2-AM) is uncharged, lipid-soluble and membrane-permeant, and is cleaved back into the parent compound by cytoplasmic esterases (Tsien, 1980). Platelets suspended in platelet-rich plasma are incubated with the ester (30 min; 37°C) which penetrates the membrane and is hydrolysed to impermeant Quin2 which is then effectively trapped within the platelet cytosol (Tsien et al., 1982c).

The available evidence indicates that the dye is distributed throughout the cytosol but not into mitochondria, lysosomes, dense tubular system or secretory granules (Tsien et al., 1985). Fluorescence levels can be calibrated in terms of $[Ca^{2+}]_i$ by exposing the dye to known $[Ca^{2+}]$ at the end of the experiment by lysing the cells and titrating the Ca^{2+} in the lysate with the chelator, EGTA. This technique reports a resting $[Ca^{2+}]_i$ in platelets of around 100nM. This is in agreement with the $[Ca^{2+}]_i$ detected in different cell types by a variety of other techniques. Rapid, concentration-dependent increases in platelet $[Ca^{2+}]_i$ have been reported in response to a range of stimulatory agonists including thrombin, PAF, vasopressin, ADP, the endoperoxide analogue U44069, 5HT and the calcium ionophores A23187 and ionomycin (Rink et al., 1982; Hallam et al., 1984a; MacIntyre et al., 1985b).

Quin2 has been used extensively in a range of cell systems to monitor changes in $[Ca^{2+}]_i$. However this technique does have some weaknesses. In particular, the relatively low fluorescence intensity of Quin2 necessitates the use of intracellular concentrations of up to 1mM (Tsien et al., 1982c). Such high intracellular concentrations inevitably result in buffering of $[Ca^{2+}]_i$ and the blunting of agonist-induced elevations in $[Ca^{2+}]_i$. Quin2 indicates increased $[Ca^{2+}]_i$ by an increase in fluorescence intensity when excited at 339nm. Absolute fluorescence intensity is difficult to standardise since it is dependent upon a number of variables including dye concentration, tissue thickness and other factors

such as lamp intensity and detection efficiency. Whilst these factors can be standardised to a large extent, it is often difficult to obtain absolute values of $[Ca^{2+}]_i$ (Tsien et al., 1985).

Tsien et al. (1985) have recently reported the development of a new dye called 'fura2' which overcomes some of the problems associated with Quin2. Fura2 gives an approximate 30-fold greater fluorescence intensity when compared with Quin2 under identical conditions. Hence, with fura2 smaller intracellular concentrations are required resulting in less buffering of $[Ca^{2+}]_i$. In addition, fura2 shifts its excitation peak upon binding Ca^{2+} from around 340 to 360nm and both bound and free species have high fluorescence intensity. Hence, the ratio of Ca^{2+}-bound to free dye can be monitored to give a measure of $[Ca^{2+}]_i$. Fura2 is less sensitive to other divalent cations (such as Mg^{2+}, Mn^{2+}, Zn^{2+} and Fe^{2+}) than Quin2 and also has a larger dissociation constant (Kd) for Ca^{2+} than Quin2. This permits the measurement of higher $Ca^{2+}{}_i$. For these and other reasons, fura2 may eventually supercede Quin2 as the dye of choice for studying $[Ca^{2+}]_i$ in platelets and other cell systems (Tsien et al., 1985).

Recently, a method has been developed for the incorporation of the calcium-sensitive photoprotein aequorin into platelets (Johnson et al., 1985). The resting platelet $[Ca^{2+}]_i$ reported by aequorin is ten-fold higher than that indicated by Quin2. This may indicate the existence of zones of elevated $[Ca^{2+}]$ within platelets which are detected by aequorin but not by

Quin2 which reports the average $[Ca^{2+}]_i$ (Johnson et al., 1985). Elevations in luminescence of platelets loaded with aequorin have been observed in response to all agonists that evoke changes in Quin2 fluorescence and by some, e.g. adrenaline and PMA, that do not (Ware et al., 1986).

Rink et al. (1982) have used Quin2 and the Ca^{2+}-ionophore ionomycin to estimate the extent of $[Ca^{2+}]_i$ required to evoke different platelet responses. They concluded that shape change, dense granule secretion and aggregation result when $[Ca^{2+}]_i$ is elevated to 0.5, 0.8 and 2μM respectively. Thrombin, however, was able to induce platelet responses at $[Ca^{2+}]_i$ below these 'threshold' levels indicating that $[Ca^{2+}]_i$ is not the sole intracellular messenger mediating thrombin-induced platelet activation. The extent of agonist-induced elevations in $[Ca^{2+}]_i$, as reported by Quin2, are reduced by around 80% when $[Ca^{2+}]_o$ (1mM) is removed by the addition of EGTA (Rink et al., 1982; MacIntyre et al., 1985c). It is concluded from this that agonist-induced elevations in $[Ca^{2+}]_i$ derive from both influx of extracellular Ca^{2+} and mobilisation of Ca^{2+} from internal stores.

The nature of the platelet internal Ca^{2+} store(s) has been extensively investigated. Around 95% of the platelet calcium content is protein-bound (Steiner & Tateishi, 1974). More than 60% of platelet calcium is in the dense granules (Murer, 1969) in a secretory pool which is not in equilibrium with the cytosol and presumably plays no role as a source of second messenger Ca^{2+}. Platelets also contain mitochondria

which can sequester calcium. The finding that secretion can occur normally in the presence of inhibitors of mitochondrial respiration suggests that mitochondrial calcium fluxes may not have any significant role in stimulus-response coupling (Friedman & Detwiler, 1975; Huang & Detwiler, 1986). It has been suggested that the platelet dense tubular system (DTS) plays a role analogous to that of the sarcoplasmic reticulum in muscle (White, 1973). This is supported by the histochemical identification of a (Ca^{2+}, Mg^{2+})-ATPase in the platelet DTS (Cutler et al., 1978). A Ca^{2+}-ATPase is a presumed requirement for any Ca^{2+}-sequestering organelle. A platelet membrane preparation capable of concentrating Ca^{2+} in an ATP-dependent manner has been described (Kaser-Glanzmann, 1977; 1979). Ca^{2+}-uptake by this membrane fraction is enhanced by cyclic AMP, possibly via cyclic AMP-dependent phosphorylation of a 22 kilodalton (kda) molecular weight protein (Kaser-Glanzmann et al., 1977; 1979). This 22 kda protein resembles phospholambam, the cyclic AMP-dependent regulator of Ca^{2+} transport in cardiac sarcoplasmic reticulum (Tada et al., 1978). Ca^{2+} uptake by the DTS is also promoted by elevated $[Ca^{2+}]_i$ and is blocked by some calmodulin antagonists (Enouf & Levy-Toledano, 1984).

At around 100nM, the resting $[Ca^{2+}]_i$ in platelets is approximately 10^4-fold less than the free $[Ca^{2+}]$ in plasma. Hence, homeostatic mechanisms must exist to maintain this low $[Ca^{2+}]_i$. This is achieved by systems which act to limit Ca^{2+} influx and promote Ca^{2+} sequestration and extrusion. Cyclic AMP may be involved in the maintenance of low $[Ca^{2+}]_i$ by impairing Ca^{2+} influx or mobilisation and/or by promoting Ca^{2+} sequestration and extrusion (Kaser-Glanzmann et al.,

1977; 1979; Feinstein et al., 1981). The mechanism of active Ca^{2+} extrusion across the plasma membrane is unknown. However, a Ca^{2+} pump similar to that present in erythrocyte membranes has recently been identified in purified human platelet plasma membrane vesicles (Enyedi et al., 1986).

The molecular mechanism underlying agonist-induced elevations in $[Ca^{2+}]_i$ are not fully understood. Generally, Ca^{2+} enters cells via voltage-operated channels (VOC) which open in response to membrane depolarisation or receptor-operated channels (ROC) which open as a direct consequence of agonist-receptor interaction. Platelets are not electrically excitable and hence, agonist-induced Ca^{2+}-influx into platelets must be mediated via ROC (MacIntyre & Rink, 1982; Doyle & Ruegg, 1985). There is evidence that agonists open divalent cation channels in the plasma membrane of human platelets (Hallam & Rink, 1985). Agonist-induced Ca^{2+} influx and mobilisation may be associated with receptor-controlled phosphoinositide metabolism (Section V.2.(3)).

V.2.(2) Mechanism of action of calcium

Calcium can act in two ways: (1) by direct combination with the target enzyme; or (2) via interaction with the ubiquitous Ca^{2+}-acceptor protein calmodulin (Cheung, 1980). The presence of calmodulin in platelets has been demonstrated (Muszbek et al., 1977) and calmodulin has been shown to be a subunit of platelet myosin light chain kinase (Dabrowska & Hartshorne, 1978; Hathaway & Adelstein, 1979). Thrombin-

induced platelet activation is associated with enhanced phosphorylation of a number of proteins. Phosphorylation of two proteins, in particular has been detected by a number of different groups. These proteins have molecular weight 20kda and 40-47kda and are referred to as the 20K and 40K proteins (Lyons et al., 1975; Haslam & Lynham, 1977). The 20K protein has been identified as the light chain of myosin and its phosphorylation is a Ca^{2+}-calmodulin-dependent reaction (Dabrowska & Hartshorne, 1978; Hathaway & Adelstein, 1979). Phosphorylation of the 20K protein was shown to correlate with secretion and may be involved in stimulus-secretion coupling (Haslam & Lynham, 1977; Haslam et al., 1979; Huang & Detwiler, 1986). Ca^{2+} and calmodulin are also involved in the regulation of other platelet enzymes including adenylate cyclase and PLA_2 (Feinstein et al., 1981).

V.2.(3) Inositol phospholipid metabolism in platelets

Inositol-containing lipids represent around 5% of the total phospholipid content of most mammalian cells (Marcus et al., 1969). Phosphatidylinositol (Ptd Ins) is the major inositol phospholipid (around 90% of total) with the more phosphorylated derivatives, phosphatidylinositol 4-phosphate (PIP) and phosphatidylinositol 4,5-bisphosphate (PIP_2) (Fig. 4) present in very small amounts. Collectively, these lipids are referred to as phosphoinositides. The main fatty acids at positions 1 and 2 respectively are stearic acid and arachidonic acid (Marcus et al., 1969). Accelerated phosphoinositide metabolism (the 'Ptd Ins response') occurs in a variety of

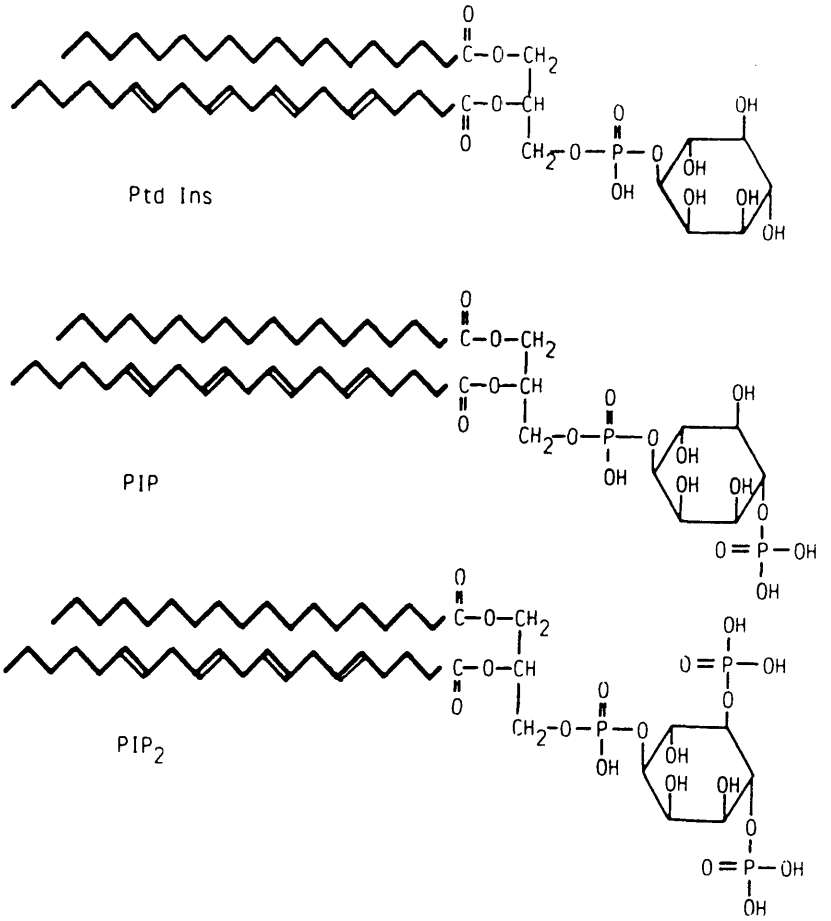

Figure 4. Structures of the phosphoinositides.

Phosphatidyl inositol (Ptd Ins), phosphatidyl inositol 4-phosphate (PIP) and phosphatidyl inositol 4,5-bisphosphate (PIP_2).

cells in response to agonist-receptor interaction (reviewed by Hokin, 1985). The initial reaction resulting from agonist-receptor combination appears to be phospholipase C-catalysed hydrolysis of PIP_2 to form inositol 1,4,5-triphosphate ($Ins(1,4,5)P_3$) and 1,2-diacylglycerol (DG) (Michell et al., 1981). Agonist-induced changes in PIP_2 levels and formation of $Ins(1,4,5)P_3$ have been reported in platelets (Agranoff et al., 1983; Siess & Binder, 1985). However, Wilson et al. (1985a) have reported that the major phosphoinositide hydrolysed by agonist-induced phospholipase C activity is Ptd Ins. Resynthesis of the phosphoinositides involves (a) the sequential dephosphorylation of the inositol phosphates to form inositol and (b) the sequential action of DG kinase and CTP Ptd OH citidyl transferase to form CDP-DG which is combined with inositol to form Ptd Ins (Fig. 5). The accepted scheme of phosphoinositide metabolism has been complicated recently by the discovery of cyclic derivatives of the inositol phosphates which are generated as a result of phospholipase C-catalysed phosphoinositide hydrolysis (Wilson et al., 1985a) and inositol phosphates containing 4,5 and 6 phosphate groups which are probably generated by sequential phosphorylation of $Ins(1,4,5)P_3$ (Batty et al., 1985; Michell, 1986).

Phosphoinositide metabolism can be monitored by measurement of the mass of intermediates (e.g. Broekman et al., 1980) or, more commonly, by measurement of radioactivity after prelabelling the cells. Platelets may be prelabelled with $[^{32}P]$-orthophosphate ($-P_i$), which is incorporated into ATP. On stimulation of phosphoinositide hydrolysis, radioactivity will be incorporated

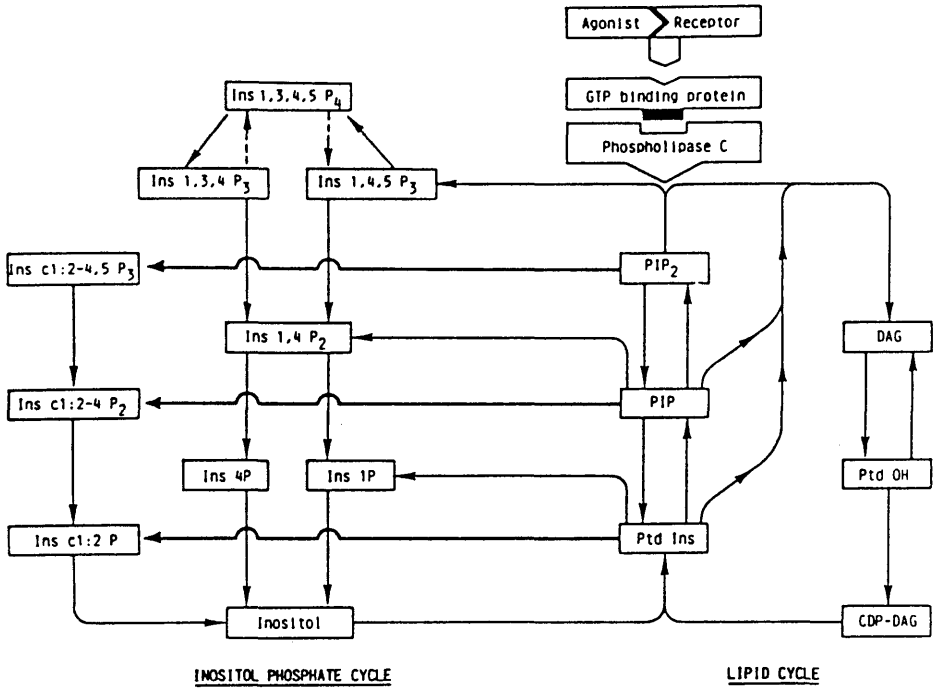

Figure 5. The inositol phospholipid cycle.

The initial receptor-controlled step is phospholipase C-catalysed hydrolysis of the phosphoinositides to yield diacylglycerol and the corresponding inositol phosphates.

Ins c1: 2-4,5 P_3 = inositol cyclic (1:2), 4,5-trisphosphate;

Ins c1: 2-4 P_2 = inositol cyclic (1:2), 4-bisphosphate;

Ins c1: 2 P = inositol (1:2) cyclic monophosphate.

into phosphatidic acid (Ptd OH) and then into Ptd Ins and a second (PIP) and third (PIP_2) labelled phosphorous will be incorporated. Initial increases in radioactivity in Ptd OH should approximate the rate of phosphoinositide turnover but after longer periods of time, a steady-state level will be approached. Phosphoinositides can also be prelabelled with ^{3}H- or ^{14}C-arachidonate or -glycerol or $[^{3}H]$ inositol. Arachidonate prelabelling allows measurement of changes in all intermediates except lysophospholipids, and the release of free arachidonate can be monitored. Lysophospholipids can be measured in cells prelabelled with glycerol. With labelled inositol, the inositol phosphates can be measured. One of the main limitations of these prelabelling methods is the presence of heterogeneous pools of precursors within platelets. Hence, quantitation may require measurement of the specific activity of the metabolites. In addition, the cyclic nature of the reactions and the existence of a number of points where label can be lost or diluted by endogenous pools make it very difficult to estimate a net flow of metabolite (Huang & Detwiler, 1986). The first evidence of a phosphoinositide response in platelets was the demonstration of agonist-induced incorporation of ^{32}P into Ptd Ins, PIP and PIP_2 in platelets prelabelled with $[^{32}P]$ -P_i (Lloyd *et al.*, 1972).

Platelet receptors for a number of stimulatory agonists are coupled to phospholipase C-catalysed phosphoinositide hydrolysis. These include 5HT ($5HT_2/S_2$ receptors), TxA_2 (TP), PAF (Paf_1), vasopressin (V_1) and thrombin (Table 1). It has recently become clear that the coupling of receptors to

phospholipase C is mediated via G proteins similar to those involved in the regulation of adenylate cyclase. This was first indicated by the demonstration that GTP could alter agonist-binding affinity of a number of receptors which are coupled to phospholipase C but not to adenylate cyclase (e.g. Goodhardt et al., 1982). This was confirmed by the observation that stable analogues of GTP stimulate phospholipase C in permeabilised platelets (Haslam & Davidson, 1984). Litosch et al. (1985) reported that 5HT-induced Ins(1,4,5)P_3 formation in blowfly salivary gland membrane preparations only occurred in the presence of GTP and was enhanced by stable analogues of GTP. This synergism between receptor agonists and guanine nucleotides demonstrates an interaction between receptors, a G protein and phospholipase C (Taylor & Merrit, 1986).

V.2.(4). Function of inositol phospholipid metabolism in platelets

Much current research has been directed towards the second messenger roles of DG (in activating protein kinase C) and Ins(1,4,5)P_3 (in mobilisation of Ca^{2+}). However, it is possible that other products of phosphoinositide metabolism, e.g. Ptd OH or lysoPtd OH, may also be involved in the control of platelet activation. Kinetic analyses have indicated that formation of DG and Ins(1,4,5)P_3 are amongst the earliest measurable events resulting from agonist-receptor interaction in a number of different cell-types (reviewed by Hokin, 1985). This is consistent with a second messenger role for these molecules.

(a) DG

The transient rise in DG levels that results from stimulus-induced phosphoinositide hydrolysis is correlated with activation of a protein kinase enzyme (Takai et al., 1979). This enzyme, called protein kinase C, is present in many tissues and is activated in the presence of Ca^{2+} and phospholipids, especially phosphatidylserine (Takai et al., 1979). DG greatly enhances the affinity of protein kinase C for Ca^{2+}, thereby activating it at resting $[Ca^{2+}]_i$.

Protein kinase C has a very wide substrate specificity when tested in vitro but its main substrate in intact platelets appears to be the 40K protein (Section V.2.(2)) (Sano et al., 1983). Protein kinase C, cyclic AMP-dependent protein kinase and Ca^{2+}-calmodulin-dependent protein kinase(s) share many phosphate acceptor proteins in vitro, but with different rates of phosphorylation at different amino acid residues (Nishizuka, 1984). The nature of the 40K protein(s) is the subject of much current study. It has been proposed that the 40K protein could be lipocortin (Touqui et al., 1986) or $Ins(1,4,5)P_3$ phosphatase (Connolly & Majerus, 1986). Lipocortin is an endogenous inhibitor of phospholipase A_2 which is inactivated by phosphorylation leading to increased phospholipase A_2 activity and liberation of arachidonic acid (Touqui et al., 1986). $Ins(1,4,5)P_3$ phosphatase is the enzyme which catalyses the inactivation of $Ins(1,4,5)P_3$.

The effects of endogenous DG can be mimicked by tumour-promoting phorbol esters such as phorbol 12-myristate 13-acetate (PMA) (Castagna et al., 1982). Addition of PMA to

intact platelets results in aggregation and secretion which is accompanied by activation of protein kinase C and phosphorylation of the 40K protein(s) (Nishizuka, 1984). PMA or synthetic DGs will synergise with the Ca^{2+}-ionophores A23187 or ionomycin to induce platelet activation (Kaibuchi et al., 1983b; Rink et al., 1983; Nishizuka, 1984). It has been proposed that both Ca^{2+}-calmodulin-dependent and protein kinase C-dependent phosphorylation reactions are necessary for full platelet activation (Kaibuchi et al., 1983b). Other possible roles for DG in stimulus-response coupling have been proposed. DG may act as a membrane fusogen to promote degranulation (Kaibuchi et al., 1983b). Moreover, the demonstration of DG-lipase in platelets suggests a possible role for DG as a source of free arachidonic acid (Bell et al., 1979).

(b) Inositol phosphates

A role for $Ins(1,4,5)P_3$ in stimulus-response coupling was indicated by its ability to induce Ca^{2+} release from an intracellular, non-mitochondrial store (Streb et al., 1983). This has been confirmed in platelets (O'Rourke et al., 1985). In platelets made permeable to small molecules by saponin pretreatment, addition of $Ins(1,4,5)P_3$ results in concentration-dependent stimulation of Ca^{2+} mobilisation (probably from the DTS), 5HT release and phosphorylation of the 20K and 40K proteins (Lapetina et al., 1984; Brass & Joseph, 1985). These findings (and the reported rapid formation of $Ins(1,4,5)P_3$) are consistent with the hypothesis that $Ins(1,4,5)P_3$ mediates agonist-induced Ca^{2+} mobilisation in platelets. Recently,

Putney (1986) also proposed a role for $Ins(1,4,5)P_3$ in mediating influx of Ca^{2+} into cells.

The major mechanism for terminating $Ins(1,4,5)P_3$ action is thought to be the removal of the 5-phosphate by specific phosphatase enzyme(s). However, it has recently become clear that $Ins(1,4,5)P_3$ may also be rapidly phosphorylated to form $Ins(1,3,4,5)P_4$ in various stimulated tissues (Batty et al., 1985; Michell, 1986). The $Ins(1,3,4,5)P_4$ is then dephosphorylated at the 5-position to form $Ins(1,3,4)P_3$. It has been suggested that the pathway for metabolism of $Ins(1,4,5)P_3$ through $Ins(1,3,4,5)P_4$ to $Ins(1,3,4)P_3$ and hence its inactivation as a calcium-mobilising ligand may be at least as important as its dephosphorylation to $Ins(1,4)P_2$ (Michell, 1986). $Ins(1,3,4,5)P_4$, $Ins(1,3,4)P_3$ and the cyclic derivatives of the inositol phosphates (Wilson et al., 1985b) may have, as yet undiscovered, roles as intracellular messengers.

(c) Other putative messengers

It has been suggested that Ptd OH or lysoPtd OH may serve as links between receptor activation and influx of Ca^{2+} by acting as ionophores in the plasma membrane (Putney, 1981; Benton et al., 1982). However, studies on the ionophoretic effects of Ptd OH in liposomes have produced negative results (Holmes & Yoss, 1983).

V.3. Arachidonic acid metabolism and platelet activation

Activation of platelets by a number of agonists is associated with liberation of free arachidonic acid (5,8,11,14-eicosatetraenoic acid). In platelets, arachidonic acid is rapidly converted to labile endoperoxides PGG_2 and PGH_2 by a cyclooxygenase enzyme. The endoperoxides, which can themselves activate platelets, are converted into the potent aggregating agent and vasoconstrictor TxA_2 (Hamberg et al., 1975). Cyclooxygenase is specifically acetylated and inhibited by aspirin (Roth et al., 1975) and this is responsible for the anti-inflammatory effects of aspirin since prostaglandins are powerful pro-inflammatory agents as well as exerting several other biological effects (reviewed by Moncada & Vane, 1979). In quiescent platelets virtually all of the arachidonic acid present is bound to membrane phospholipids (particularly phosphatidylcholine and Ptd Ins) esterified to glycerol at glycerol sn-2. Agonist-induced liberation of free arachidonic acid from complex phospholipids is the rate-determining step in eicosanoid formation (Rittenhouse-Simmons & Deykin, 1981). This can be achieved by activation of a phospholipase A_2 enzyme which cleaves phospholipids at the sn-2 position. Free arachidonic acid may also be liberated as a consequence of stimulated phosphoinositide metabolism. Rittenhouse-Simmons & Deykin (1981) proposed that DG liberated from the phosphoinositides (by phospholipase C action) is hydrolysed by DG lipase to release free arachidonic acid whilst Majerus et al. (1983) suggested that DG is first converted to monoacylglycerol (by DG lipase action at sn-1) before arachidonic acid is liberated

by the action of monoglyceride lipase. Conversion of free arachidonic acid to PGG_2, PGH_2 and TxA_2 provides a positive feedback mechanism to promote platelet activation.

VI. Aims

The aims of this study were to investigate the effects of agonists and agonist combinations on several platelet responses and transduction processes including:-

(a) shape change

(b) aggregation in whole blood, platelet-rich plasma and gel-filtered platelet suspensions

(c) dense granule release

(d) $[Ca^{2+}]_i$

(e) phosphoinositide hydrolysis

(f) cyclic AMP content.

By examining the effects of agents, alone and in combination, on platelet responsiveness and on the second messenger systems (using washed platelet suspensions), I attempted to investigate:-

(1) The roles of $[Ca^{2+}]_i$ and phosphoinositide hydrolysis in mediating platelet activation induced by adrenaline, ADP, vasopressin and PAF.

(2) The roles of $[Ca^{2+}]_i$, phosphoinositide hydrolysis and activation of protein kinase C in mediating the potentiation of agonist-induced platelet activation by adrenaline.

(3) The mechanism of inhibition of platelet responsiveness by agents which elevate cyclic AMP content and the relative susceptibility of different responses to inhibition by cyclic AMP.

(4) The effects of stimulatory agonists and of activation of protein kinase C on platelet cyclic AMP metabolism.

The responses of platelets to combinations of agents (both stimulatory and inhibitory) in the more physiological milieu of whole blood were examined by measuring platelet aggregation in this medium.

I also attempted to develop a simply assay for cyclic AMP-dependent protein kinase activity in crude platelet extracts which would allow the effects of platelet stimulatory and inhibitory agonists on this enzyme to be determined. The rationale behind this study was: (a) to examine the relationship between activation of cyclic AMP-dependent protein kinase and inhibition of the various platelet responses and (b) to investigate the controversial proposition that agonist (particularly adrenaline)-induced platelet activation could result from a decrease in the basal level of cyclic AMP, perhaps in a specific subcellular pool, by examining the effects of adrenaline and other stimulatory agonists on cyclic AMP-dependent protein kinase activity.

MATERIALS AND METHODS

I.1. Materials

The drugs and reagents used in the course of this project are listed below and the sources indicated. Except where stated, reagents were dissolved in glass-distilled water and further dilutions were made using 0.9 % saline. Adenosine 5'-diphosphate (ADP); ATP; adenosine 3',5'-cyclic monophosphate (cyclic AMP); 8 bromo-cyclic AMP; 8-arginine vasopressin; bovine serum albumin; HEPES; apyrase (grade III); creatine phosphate; creatine phosphokinase; histone (type IIA); 3-isobutyl 1-methyl xanthene (IBMX); ethylene glycol bis-(β-aminoethyl ether) N,N,N',N'-tetraacetic acid (EGTA); phorbol 12-myristate 13-acetate (PMA); 4αphorbol 12,13-didecanoate (4αPDD); sepharose 2B and dimethylsulphoxide (DMSO) were all obtained from Sigma (U.K.). EGTA was dissolved in 0.4N NaOH at a concentration of 0.4M (pH < 8). PMA and 4αPDD were dissolved in DMSO. Quin2-acetoxymethyl ester (Quin2-AM) was obtained from Lancaster Synthesis (Morecambe, England) and was dissolved in DMSO. 2-acetyl-1-alkyl-sn-glycero-3-phosphocholine (platelet-activating factor, PAF) was from Bachem (Bubbendorf, Switzerland) and was dissolved at 1 mg per ml in 0.9% saline containing 0.25% BSA, with subsequent dilutions in saline alone. Adrenaline-tartrate was purchased from Koch-Light Laboratories (U.K.) and ionomycin (dissolved in DMSO) was from Calbiochem. Prostaglandin I_2 (PGI_2), PGE_1, 6 keto-PGE_1 and PGD_2 were gifts from Dr J.E. Pike (Upjohn, U.S.A.). PGI_2 was dissolved in Tris-HCl buffer, pH 9.2. The other prostaglandins were dissolved initially in ethanol and diluted with 9 volumes of Na_2CO_3

(3 mM) to give 3.3 mM stock solutions. Further dilutions were made in saline. The heat-stable inhibitor of cyclic AMP-dependent protein kinase was obtained from Sigma and a more purified preparation of this was a gift from Dr J.D. Scott, Howard Hughes Medical Institute, Research Laboratories, Seattle.

Adenosine 3',5'-cyclic phosphoric acid 2'-O-succinyl-3-[^{125}I] iodotyrosine methyl ester ([^{125}I]-cyclic AMP; 600 Ci per mMole) and adenosine 5'-[γ-^{32}P] triphosphate, triethylammonium salt ([^{32}P]-ATP; 20.8 Ci per mMole) were from Amersham International (Amersham, U.K.). Carrier-free [^{32}P]-orthophosphate (P_i) was supplied by the Regional Isotope Dispensary, Western Infirmary, Glasgow, U.K.

1.2. Buffers

Buffer A (Hepes-buffered Tyrodes solution):-

NaCl	-	129 mM
trisodium citrate	-	10.9 mM
$NaHCO_3$	-	8.9 mM
dextrose	-	0.56 mM
Hepes	-	5 mM
KCl	-	2.8 mM
KH_2PO_4	-	0.8 mM
$MgCl_2$	-	0.84 mM
$CaCl_2$	-	2.4 mM
bovine serum albumin	-	0.35% (w/v)

The above were dissolved in glass-distilled water and the pH was adjusted to 7.4 using NaOH (1N).

Buffer B (calcium-free, phosphate-free buffer):-

NaCl	-	150 mM
KCl	-	4 mM
$MgCl_2$	-	1 mM
dextrose	-	10 mM
Hepes	-	5 mM

bovine serum albumin - 0.35%

The above were dissolved in glass-distilled water and the pH was adjusted to 7.4 using HCl (1N).

Buffer C (100 mM potassium phosphate buffer):-

K_2HPO_4 (200 mM)	-	66.25 ml
KH_2PO_4 (200 mM)	-	183.75 ml

Glass distilled water - 250 ml

The above were mixed and the pH was adjusted to 6.4 using HCl (10N).

Buffer D

NaCl	-	129 mM
$NaHCO_3$	-	8.9 mM
KCl	-	2.8 mM
K_2HPO_4	-	0.81 mM
MgCl	-	0.84 mM
dextrose	-	0.56 mM

The above were dissolved in glass-distilled water and the pH was adjusted to 7.4 using NaOH (1N).

Buffer E (62 mM Potassium phosphate buffer)

K_2HPO_4 (125 mM) - 122.5 ml

KH_2PO_4 (125 mM) - 127.5 ml

Glass-distilled water - 250 ml

The above were mixed and the pH was adjusted to 6.8 using HCl (10N).

II. Preparation of platelets

II.1 Preparation of platelet-rich plasma (PRP)

Blood was obtained by antecubital venepuncture from healthy human volunteers who denied taking drugs known to affect platelet function. Blood was collected into 10% (volume/volume, v/v) trisodium citrate (0.13 molar, M) and centrifuged (1000g; 5 minutes; room temperature) to sediment erythrocytes and other blood cells. The supernatant PRP was aspirated (Pollock et al., 1984).

II.2. Isolation of platelets

(a) Gel-filtration.

Columns of sepharose 2B (50-100 ml bed volume) were equilibrated with buffer A. PRP was layered onto the column and when this had entered the gel, further buffer was added. The column eluate was collected. The platelets (now suspended in buffer A; 2×10^8 platelets per ml) eluted immediately after the dead volume and preceded the plasma proteins and other plasma constituents (Pollock et al., 1984).

(b) PGI_2 Centrifugation

PRP was centrifuged (800g; 10 minutes; room temperature) in the presence of PGI_2 (0.3 μM) to prevent activation. The supernatant plasma was decanted and the platelet pellet re-suspended in buffer B (see Section II) to give a final platelet count of approximately 3×10^9 per ml (MacIntyre & Pollock, 1983).

(c) Low $[Ca^{2+}]$ /low pH centrifugation

PRP was treated with EGTA (4 mM) to chelate extracellular Ca^{2+} ions and the pH was adjusted to 6.8 with citric acid (10 μM) to prevent platelet activation during centrifugation (800g; 10 min; room temperature). The supernatant plasma was decanted and the platelet pellet was resuspended in one tenth volume of buffer D to give a platelet count of approximately 3×10^9 per ml.

III. Platelet Function Assays

III.1 Shape change

Shape change was monitored photometrically using PRP or gel-filtered platelets. This involved measuring the decrease in light transmission that followed agonist addition to stirred (700 rev/min) platelet suspensions (0.6 ml; 37°C) and was monitored using a Mallin clinical aggregation recorder. In these studies, to reduce extracellular Ca^{2+} and hence prevent aggregation, EGTA (4 mM) was added to each platelet sample 60 seconds before agonist addition (Born, 1962; Michal & Born, 1971).

III.2. Secretion

Secretion of platelet dense granule constituents was monitored by measuring ATP release using firefly luciferin-luciferase luminescence. This was measured simultaneously with aggregation in stirred (700 rev/min) platelet samples (0.45 ml; 37°C) by using a Chronolog Corporation luminescence aggregometer (Feinman et al., 1977).

III.3.(a) Photometric (turbidometric) measurement of aggregation.

Aggregation was monitored in PRP and in gel-filtered platelets by measuring increases in light transmission through stirred (700 rev/min) platelet suspensions using either a Chronolog Corporation luminescence aggregometer (0.45 ml samples) or a Mallin clinical aggregation recorder (0.6 ml samples) (Born, 1962).

III.3.(b) Single platelet counting method for monitoring aggregation.

Platelet aggregation was monitored in whole blood by an electrical impedence method. By utilising the Clay Adams Ultra-Flow 100 whole blood platelet counter, platelet numbers before and after addition of an aggregating agent were counted electronically without the need to separate platelets from other blood cell types (Day et al., 1980). Immediately following blood collection, an initial platelet count was obtained from the 'stock' citrated blood. Aliquots of blood (0.5 ml) were transferred to polystyrene vials which, where stated, contained aspirin to give a final concentration of 2 mM. The vials were gassed with a mixture of 5% CO_2 in air,

capped and placed into racks in a shaking water bath (Grant Model SS30; 37°C; 90-100 shakes/min). After 30 min., a 10 µl sample was taken from each aliquot, mixed with a pre-filled diluent pot (Clay Adams) and duplicate control counts were taken and the mean count recorded. An aggregating agent was then added to the blood, the tube was re-gassed, capped and returned to the water bath. Further 10 µl samples were taken for counting at standard times ($\geq$ 1 min) after agonist addition (Lumley & Humphrey, 1981).

III.3.(c) Comparison of photometric and electronic methods for monitoring platelet aggregation.

These studies involved removing 10 µl aliquots of PRP from aggregometer cuvettes before and at regular intervals (30 sec - 5 min) after agonist addition for the electronic measurement of platelet count. Each 10 µl aliquot was mixed with a pre-filled diluent pot (Clay Adams) containing formaldehyde (1%) and platelet count was determined later (1-2 hours) using the Clay Adams platelet counter. Thus, changes in light transmission monitored in the aggregometer could be correlated with changes in platelet count.

IV. Platelet Cytosolic Free Calcium Ion Concentration: $[Ca^{2+}]_i$

Platelet $[Ca^{2+}]_i$ was monitored using the fluorescent quinoline dye, Quin 2, which displays high affinity for Ca^{2+} and undergoes fluorescent changes as a result of Ca^{2+} binding (Rink et al., 1982; Tsien et al., 1982a,b,c). PRP was incubated with Quin 2 acetoxymethyl ester (10 µM; 30 min; 37°C)

which is membrane permeant and crosses the plasma membrane where it is rapidly hydrolysed by cytosolic esterases to form the hydrophilic polycarboxylate anion that is Quin 2. Quin 2 is thus trapped within the cytosol of intact platelets and can report the resting $[Ca^{2+}]_i$ and the changes which result from exposure to agonists. Quin 2-loaded platelets were separated from plasma containing extraneous dye by gel-filtration (Section I.2.(a)). The Quin 2 content of the platelets was around 1 mmole/litre of cell water. Gel-filtered platelets were stored at 37°C in stoppered plastic tubes. Immediately before use, the external free Ca^{2+} concentration $[Ca^{2+}]_o$ was adjusted either to 1 mM by the addition of $CaCl_2$ or to 1-10 nM by the addition of EGTA (4 mM). Platelets (0.5 ml) were placed in 1 cm square quartz cuvettes and platelet count was adjusted to 5×10^7 platelets/ml by the addition of 1.5 ml of buffer A. Fluorescence was monitored in a Perkin-Elmer LS3 Fluorescence Spectrometer with standard monochromater settings of 339 nm - excitation and 492 nm - emission (MacIntyre *et al.*, 1985c). $[Ca^{2+}]_i$ was calculated from the observed fluorescence according to the following equation:-

$$[Ca^{2+}] = Kd \times (F - F_{min})/(F_{max} - F),$$

where Kd is the effective dissociation constant of Quin 2 for Ca^{2+} (Kd = 115 nM under these conditions); F is the relative fluorescence reading and F_{max} and F_{min} are the fluorescences at very high and very low $[Ca^{2+}]$ respectively (Tsien *et al.*, (1982a). F_{max} was obtained by the addition of 50 μM digitonin

to lyse the platelets and expose all of the Quin 2 to the extracellular Ca^{2+} and to liberated intracellular Ca^{2+} i.e. Ca^{2+} > 1 mM, and F_{min} was obtained by the later addition of EGTA (4 mM) to chelate all of the Ca^{2+} (Rink et al., 1982).

V. Inositol Phospholipid Studies

Inositol phospholipid studies were performed by Mr A. McNicol essentially as described by MacIntyre & Pollock (1983). Agonist-induced hydrolysis of inositol phospholipids was monitored by measuring the formation of $[^{32}P]$-Ptd OH in platelets prelabelled with $[^{32}P]$-orthophosphate ($-P_i$). Platelet suspensions (prepared by centrifugation; Section II.2.(b)) were incubated (37°C; 90 min) with carrier-free $[^{32}P]$-P_i (30 μCi/ml). Platelets were then pelleted by centrifugation (800g; 10 min; room temperature) in the presence of PGI_2 (0.3 μM) and re-suspended in 1.5 times the labelling volume of fresh buffer B. Radiolabelled platelets (0.4 ml containing 0.5 - 1 mg protein) were dispensed into plastic tubes at 37°C. Reactions were initiated by the addition of agonist(s) or vehicle and terminated by transferring the entire platelet sample into a glass test-tube containing 2 ml of chloroform/methanol/10M-HCl (25:50:4, by volume) at room temperature (Lloyd et al., 1972; MacIntyre & Pollock, 1983). Platelet lipids were extracted (Lloyd et al., 1972), dried at 40°C under nitrogen, separated by two-dimensional thin layer chromatography (Yavin & Zutra, 1977) and detected by exposure to iodine vapour and/or autoradiography on Ilford 25 EP X-ray film (15-20 hours). The spots corresponding to Ptd OH were scraped into vials and counted for radioactivity

by liquid scintillation spectrometry. Under these conditions, the platelets were functionally viable (MacIntyre & Pollock, 1983). Changes in $[^{32}P]$-Ptd OH reflect changes in Ptd OH concentration (Holmsen et al., 1984) and the DG precursor of Ptd OH is most probably derived via hydrolysis of phosphoinositides (MacIntyre & Pollock, 1983).

VI. Platelet Cyclic AMP Content

Cyclic AMP was extracted from gel-filtered platelet samples (0.4 - 1 ml) by the addition of two volumes of ethanol. The samples were vortex-mixed, left at room temperature for 5 min - 1 hour and then centrifuged (500g; 10 min; room temperature) and the supernatant, ethanol-soluble fraction, was decanted and evaporated at 60°C. The dried samples were resuspended in sodium acetate buffer (50 mM; pH 5) to give a cylcic AMP concentration in the range of 0.01 - 50 pmoles/100 µl. The samples were then stored at -20°C prior to assay for cyclic AMP. The cyclic AMP concentration was estimated by radioimmunoassay essentially as described by Brooker et al. (1979). Authentic cyclic AMP standard concentrations (0.01 - 100 pmoles/100 µl; triplicate samples) were prepared in sodium acetate buffer. To maximise assay sensitivity, samples (100 µl) were acetylated by the addition of 5 µl of a freshly made mixture of acetic anhydride and triethylamine, 1:2 by volume (Frandsen & Krishna, 1976) and the samples were rapidly vortex-mixed. To each tube was added 150 µl of a 1/10,000 dilution (in 0.1% bovine serum albumin) of anti-cyclic AMP antiserum (raised in goats against human serum albumin conjugated to succinyl cyclic AMP) and 25 µl of a 1/175 dilution of $[^{125}I]$-cyclic AMP

(approximately 3000 cpm/sample). Samples were mixed and incubated for 16 hours at 4°C. After this incubation period, 0.5 ml of a suspension of washed charcoal (1%, w/v) in Buffer C containing 0.25% bovine serum albumin (4°C) was added. The samples were mixed and the charcoal sedimented by centrifugation (4000g; 4 min; 4°C). Aliquots (0.5 ml) of the supernatant were taken to assess antibody-bound $\left[^{125}I\right]$-cyclic AMP which was counted (5 min) in an EMI gamma counter. Standard calibration curves in each experiment were plotted as radioactivity bound (cpm) against concentration of cyclic AMP or as B/B_0 against concentration of cyclic AMP where B and B_0 represent the amount of $\left[^{125}I\right]$-cyclic AMP bound in the presence and absence, respectively, of unlabelled cyclic AMP. In the absence of unlabelled cyclic AMP, approximately 30% of the total added $\left[^{125}I\right]$-cyclic AMP was bound. Amounts of cyclic AMP in unknown samples were determined by reference to the calibration curve. The specificity of the anti-serum was tested by measuring the ability of a range of adenine and guanine nucleotides to compete with $\left[^{125}I\right]$-cyclic AMP for binding to the anti-serum. As illustrated in Figure 6, the anti-serum is relatively specific for cyclic AMP with 10^3-fold (cyclic GMP), 10^4-fold (AMP, ADP, ATP) and 10^5-fold (adenosine) higher concentrations of the other nucleotides required to produce 50% displacement of the $\left[^{125}I\right]$-cyclic AMP.

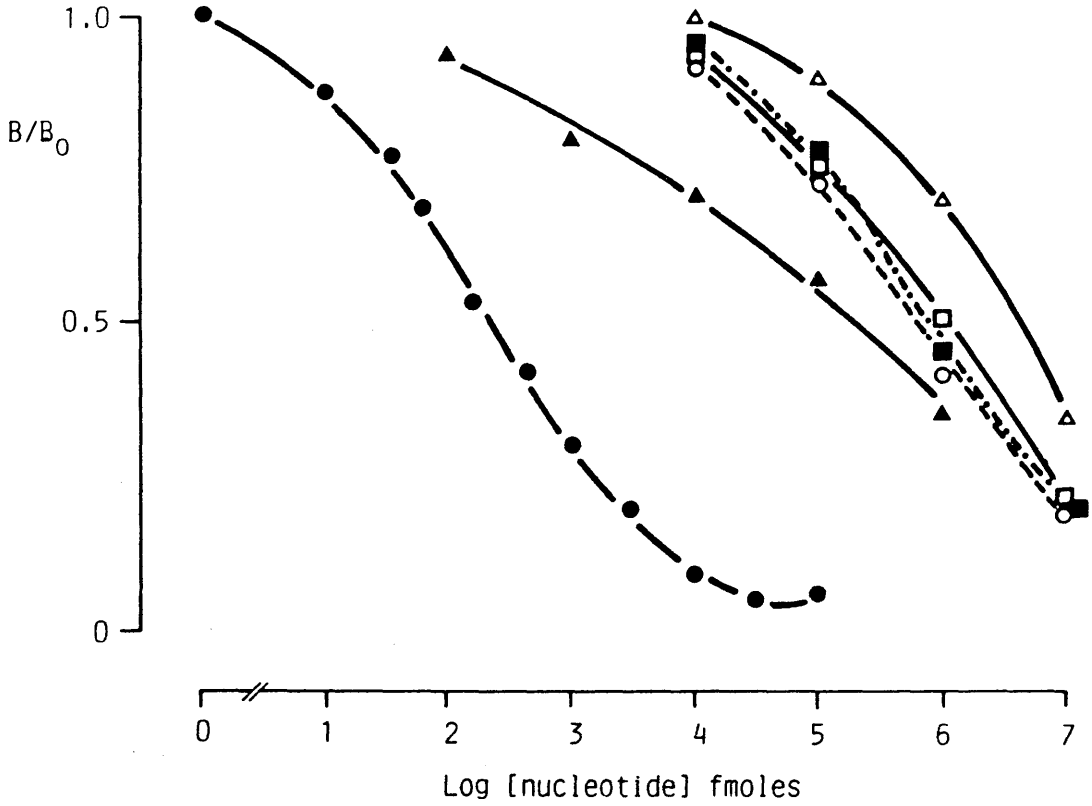

Figure 6. Effects of a range of nucleotides on the binding of [^{125}I]-cyclic AMP to anti-cyclic AMP antiserum.

The ability of the acetylated forms of cyclic AMP (●); cyclic GMP (▲); ATP (o); ADP (□); AMP (■) and adenosine (△) to compete with [^{125}I]-cyclic AMP for binding to the anti-cyclic AMP antiserum was measured as described in Methods Section.

VII. Preliminary studies of cyclic AMP-dependent protein kinase activity in crude platelet extracts

VII.1. Preparation of platelet particulate and soluble fractions

Platelet suspensions were prepared by low $[Ca^{2+}]$ /low pH centrifugation. Aliquots (0.12 ml) were incubated (1-5 min; 37°C) with saline or modulators of platelet adenylate cyclase activity. After this incubation period, 20 µl samples were removed for the estimation of cyclic AMP content and 400 µl of ice-cold buffer E containing EGTA (4 mM) was added to the remaining platelet sample. The samples were then rapidly frozen by immersion in a mixture of solid CO_2/acetone and then thawed in a water bath at 37°C with shaking. The samples were re-frozen and thawed a further three times. All further manipulations were carried out at 4°C. Any remaining unlysed platelets were pelleted by centrifugation (1000g; 5 min; 4°C) using a 'Koolspin' refrigerated centrifuge (Burkard). The supernatant was decanted into labelled thick-walled ultra-centrifuge tubes (Beckman, U.K.). Soluble and particulate fractions were separated by centrifugation (100,000g; 30 min; 4°C) using a Beckman ultra-centrifuge. Platelet particulate fractions were resuspended in 0.3 ml of a potassium phosphate buffer (100 mM; pH 6.8; 4°C). Platelet soluble and particulate fractions were assayed for protein kinase activity within 30 min of preparation.

VII.2. Protein kinase assay

Cyclic-AMP-dependent protein kinase activity was determined by the filter paper assay procedure described by Corbin (1983). In principle, the assay is based upon the ability of cyclic

AMP-dependent protein kinase to catalyse the incorporation of ^{32}P from $[\gamma\text{-}^{32}P]$-ATP onto a protein substrate (mixed histone, Sigma type IIA):-

$$Mg^{2+}.ATP + HISTONE \longrightarrow Mg^{2+}.ADP + HISTONE\text{-}^{32}P$$

The phosphorylated histone is then trapped by precipitation onto filter paper. Assay mixtures (final volume 0.25 ml) contained Mg.acetate (2.4 mM); histone (8 mg per ml); $[\gamma\text{-}^{32}P]$-AT (0.14 mM, around 10 cpm per pmole) with or without cyclic AMP (4 µM). The composition of this assay mixture was varied and other reagents were included in these preliminary experiments in an attempt to optimise the assay conditions. The reaction was initiated by the addition of 0.1 ml of platelet soluble or particulate fraction to the mixtures which were incubated for up to 10 min at 30°C. At the end of this incubation period, an excess of unlabelled ATP (50 µl of 40 mM) was added to the reaction mixtures and the samples were transferred to ice to effectively stop the reaction. A sample (0.1 ml) of the mixture was then spotted onto filter paper disks (Whatman, 3 MM, 2.4 cm diameter) numbered with pencil lead. The disks were left at room temperature for 15-30 min to allow all of the histone to be absorbed. They were then dropped into a stainless steel basket located in a beaker containing 10% TCA (around 5 ml per disk) on ice. The basket was constructed so that space was provided for a magnetic stirr bar underneath the basket (Corbin & Reimann, 1974). The filters were washed for 15 min at 4°C with stirring. The TCA was then replaced with fresh 10% TCA at room temperature and this washing procedure was repeated a further three times. The filter disks were

then washed in 95% ethanol and then diethyl-ether for 5 min each. The disks (which should now contain only histone-bound radioactivity) were dried for around 5 min using a hair-dryer and then placed in a vial containing 10 ml scintillant. The radioactivity bound to the histone substrate was determined by liquid scintillation counting using a Packard Tri-carb Liquid Scintillation Spectrometer. To correct for phosphorylation of endogenous platelet protein substrates, a blank containing no histone was included in each assay and this value was subtracted from the other samples.

Results are expressed as units of kinase activity or as cyclic AMP-dependent protein kinase activity ratio. A unit is defined as the amount of enzyme activity catalysing the transfer of 1 pmole of phosphate from $[\gamma\text{-}^{32}P]$-ATP onto a histone mixture in 1 min at 30°C (Corbin & Reimann, 1974). The activity ratio is a measure of the fraction of the enzyme in the active form:-

$$R_2C_2 + 4 \text{ cyclic AMP} \rightleftharpoons R_2 \cdot \text{cyclic AMP}_4 + 2C$$

inactive — active

$$\text{ACTIVITY RATIO} = C/(C + R_2C_2)$$

The activity ratio is obtained by dividing the activity detected in the absence of added cyclic AMP (i.e. C) by the total activity which is detected in the presence of an excess of cyclic AMP, 4 μM (i.e. $C + R_2C_2$; Corbin & Reimann, 1974).

VIII. Protein determination

The protein content of platelet samples was determined by the method of Lowry et al. (1951).

IX. Analysis of results

Experiments were normally performed in triplicate and each experiment was performed at least three times. Results are expressed as mean values $\pm$ standard error of the mean (SEM). Groups of data were compared statistically using Student's t-test and probability (P) values of less than 0.05 were taken to be significant.

RESULTS

I. Platelet responses to addition of single agonists

The effects of adrenaline, ADP, vasopressin and PAF on a range of platelet functions and putative transduction processes were examined. The responses monitored were:-

1. aggregation in whole blood,
2. aggregation in PRP,
3. aggregation and ATP secretion in Quin 2-loaded, gel-filtered platelets,
4. changes in $[Ca^{2+}]_i$,
5. inositol phospholipid hydrolysis.

I.1. Agonist-induced platelet aggregation in whole blood

The main agonists utilised in the course of this study were adrenaline, ADP, vasopressin and PAF, each of which induced concentration-dependent reductions in single platelet count (i.e. platelet aggregation) in whole blood. Adrenaline-induced aggregation reached a peak approximately 10-15 min after agonist addition with a maximum 70% reduction in platelet count (Fig. 7A). In comparison, ADP-, PAF- and vasopressin-induced aggregation were more rapid (peak within 1-2 min) and transient and these agonists induced up to 80% reductions in platelet count (Fig.7B-C). In some cases (14/24 individuals) vasopressin induced a maximum reduction in platelet count of less than 50% (Fig. 8). Platelets from these individuals consistently failed to respond to vasopressin when tested on different days. Responses to adrenaline, ADP, vasopressin and PAF were not significantly affected by incubation (30-90 min) with aspirin (2 mM), not shown.

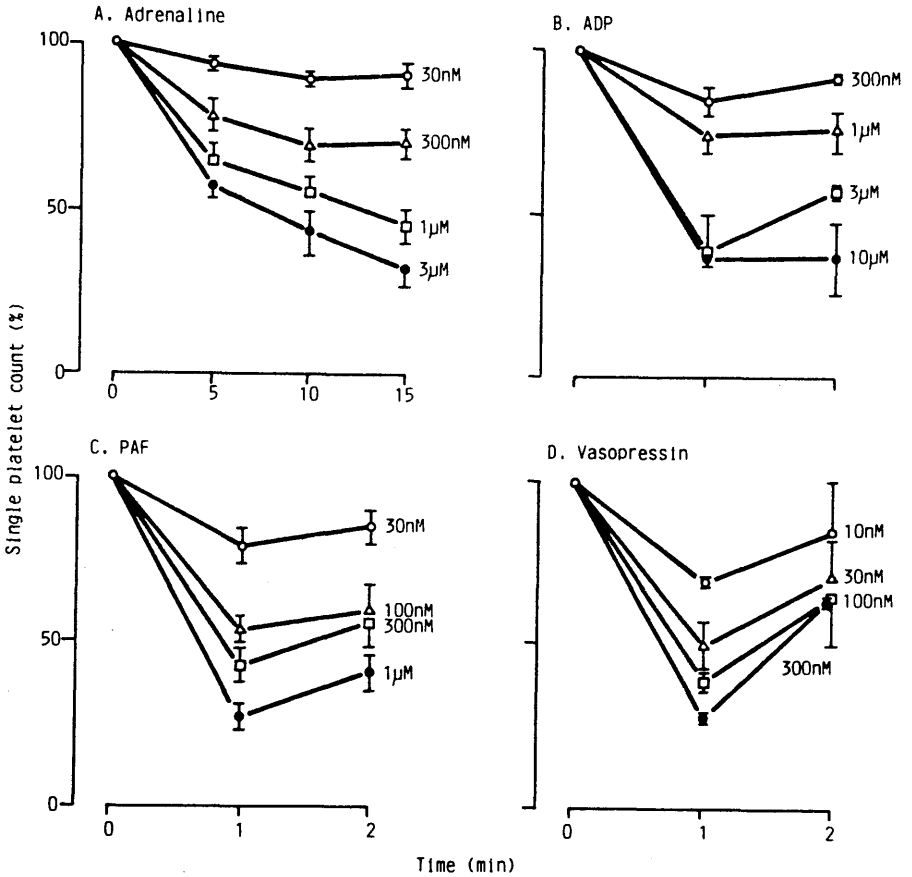

Figure 7. Agonist-induced platelet aggregation in whole blood.

Citrated human whole blood samples were prepared and agonist-induced reductions in single platelet count were monitored as described in Methods Section. Platelet count was measured before and at the indicated times after addition of (A) adrenaline: 30 nM (o), 300 nM (Δ), 1 μM (□) and 3 μM (●); (B) ADP: 300 nM (o), 1 μM (Δ), 3 μM (□) and 10 μM (●); (C) PAF: 30 nM (o), 100 nM (Δ), 300 nM (□) and 1 μM (●); (D) vasopressin: 10 nM (o), 30 nM (Δ), 100 nM (□) and 300 nM (●). Data are means and standard errors from four to six experiments using blood from different donors.

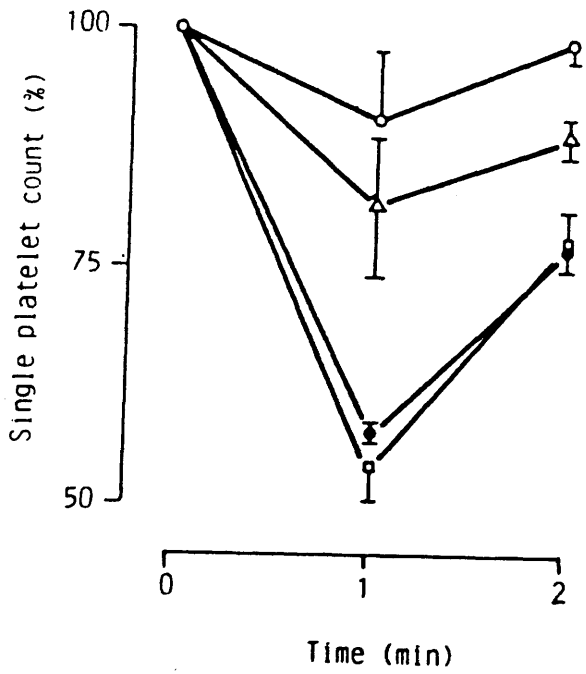

Figure 8. Vasopressin-induced decreases in whole blood platelet count - poor responses seen in some individuals.

Changes in whole blood platelet count were monitored in response to addition of vasopressin: 10 nM (o), 100 nM (Δ), 1 μM (□) and 10 μM (●). Data are the means and standard errors from four experiments using blood from different donors.

I.2. Agonist-induced platelet aggregation in PRP: a comparison of turbidometric and electronic methods for monitoring platelet aggregation

Agonist-induced changes in light transmission and reductions in single platelet count were monitored simultaneously in PRP as described in Methods (Section III.3). The aim of this study was to compare the sensitivity of the turbidometric and single platelet counting methods for monitoring platelet aggregation. Incubation with 10^{-7}M adrenaline resulted in a significant reduction in platelet count with very little change in the light transmission recording (Fig. 9A, B). Increasing concentrations of adrenaline (up to 3×10^{-6}M) resulted in greater and more rapid reductions in platelet count and significant increases in light transmission (Fig. 9). The concentration-response relationships for maximum adrenaline-induced reductions in platelet count and increases in light transmission are shown in Figure 10. The electronic single platelet counting technique provided a more sensitive index of aggregation (EC_{50}, i.e. concentration producing 50% of maximum response, $\sim 3 \times 10^{-7}$M) than the light transmission measurements ($EC_{50} \sim 2 \times 10^{-6}$M). Similarly, ADP- (Figs. 11 & 12), vasopressin - (Figs. 13 & 14) and PAF- (Figs. 15 & 16) induced reductions in single platelet count could be detected in the absence of significant increases in light transmission. Reductions in platelet count of up to 50% occurred without significant changes in light transmission. In each case the EC_{50} values for agonist-induced reductions in single platelet count were significantly less than the corresponding EC_{50} values for agonist-induced increases in light transmission

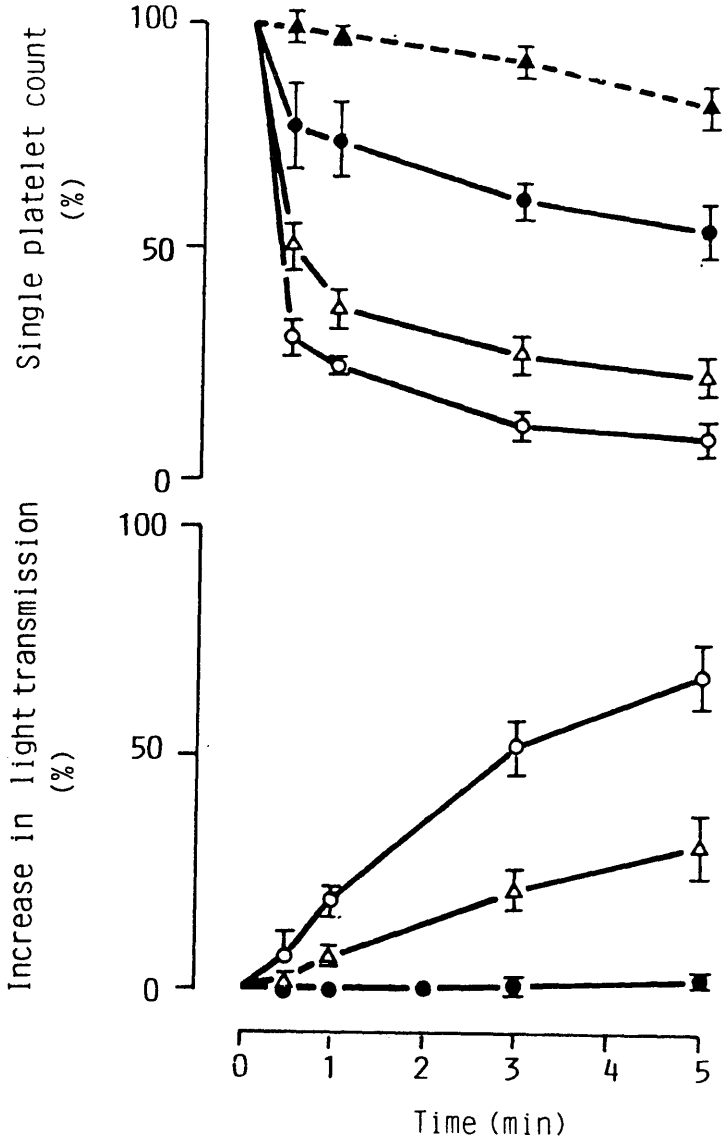

Figure 9. Platelet-rich plasma: adrenaline-induced reductions in platelet count and increases in light transmission.

Platelet-rich plasma was prepared, aggregation was monitored photometrically and samples were removed at the times indicated for the determination of platelet count as described in Methods Section. Aggregation was induced by 100 nM (●), 1 μM (△) and 3 μM (o) adrenaline or vehicle (▲). Data are the means and standard errors from six experiments.

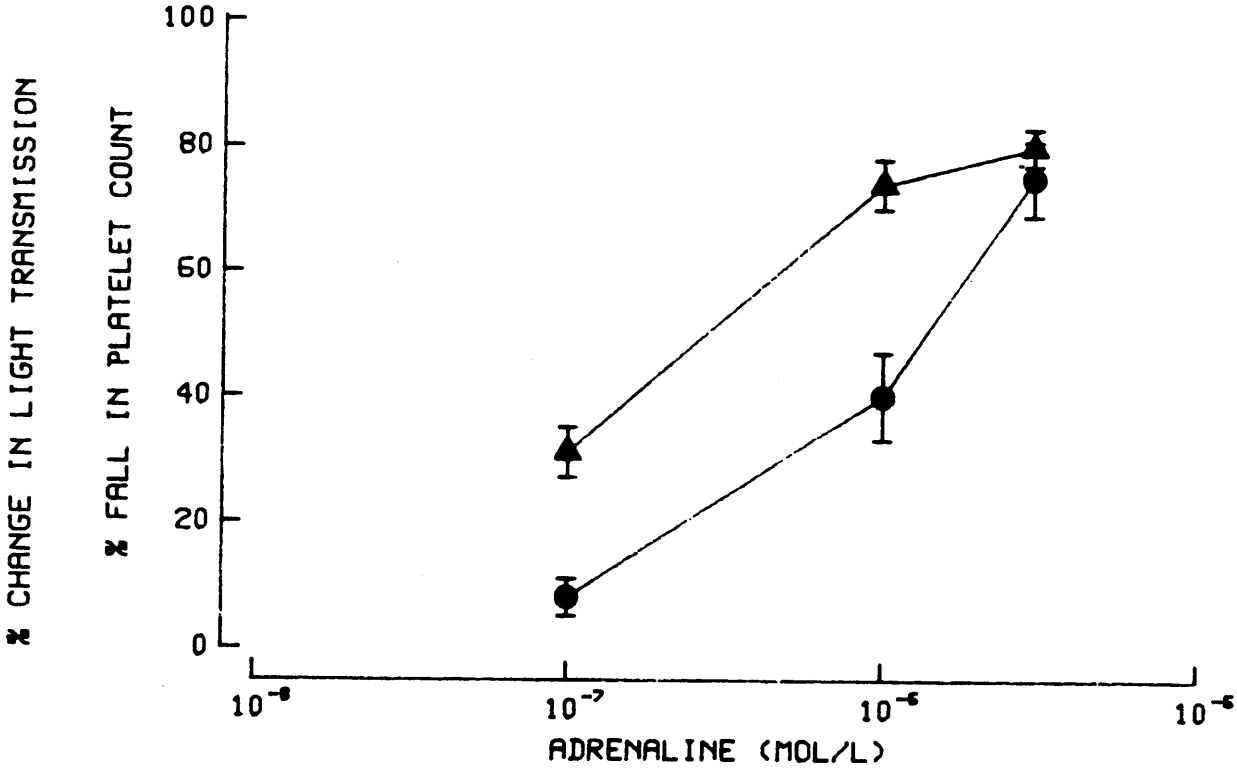

Figure 10. Platelet-rich plasma: comparison of concentration-effect relationships for maximal adrenaline-induced increases in light transmission and reductions in platelet count.

The data shows the peak increase in light transmission (●) and reduction in platelet count (▲) induced by adrenaline at the concentrations indicated. Data are the means and standard errors from six experiments.

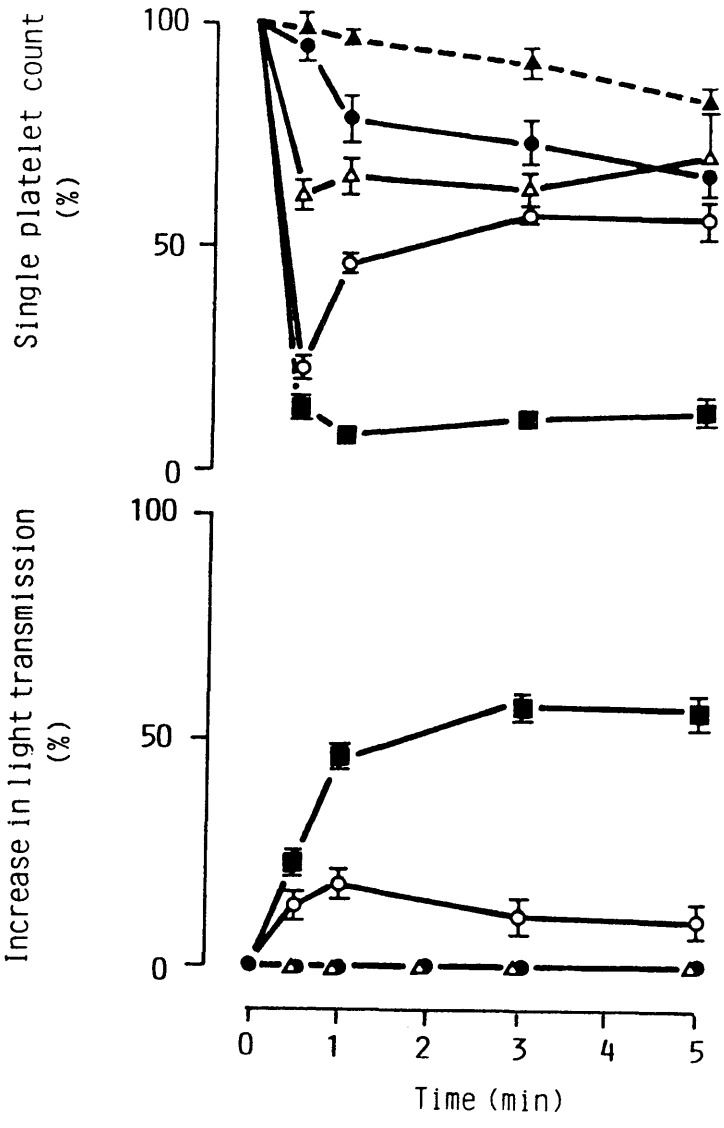

Figure 11. Platelet-rich plasma: ADP-induced reductions in platelet count and increases in light transmission.

Platelet-rich plasma was prepared, aggregation was monitored photometrically and samples were removed at the times indicated for the determination of platelet count as described in Methods Section. Aggregation was induced by 100 nM (●), 300 nM (△), 1 μM (o) and 3 μM (■) ADP or vehicle (▲). Data are the means and standard errors from four different experiments.

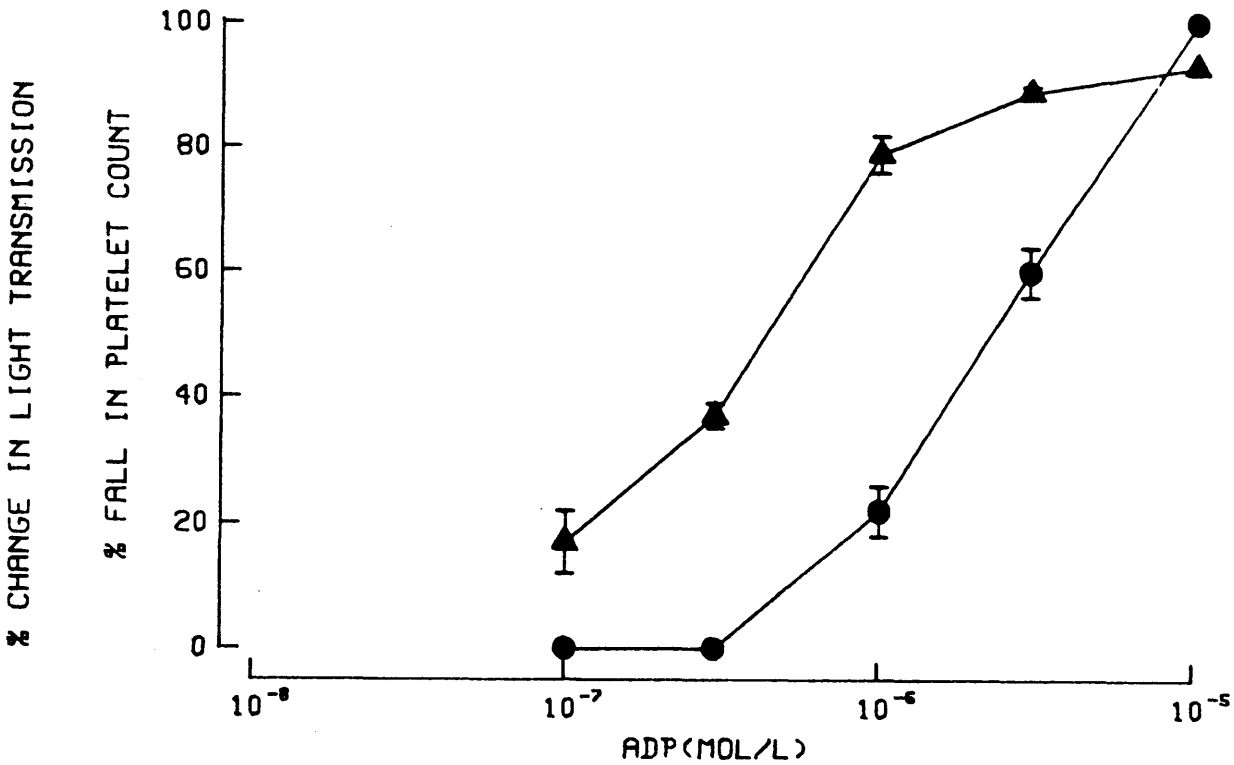

Figure 12. Platelet-rich plasma: comparison of concentration-effect relationships for maximal ADP-induced increases in light transmission and reductions in platelet count.

The data shows the peak increases in light transmission (●) and reductions in platelet count (▲) induced by ADP at the concentrations indicated. Data are the means and standard errors from four different experiments.

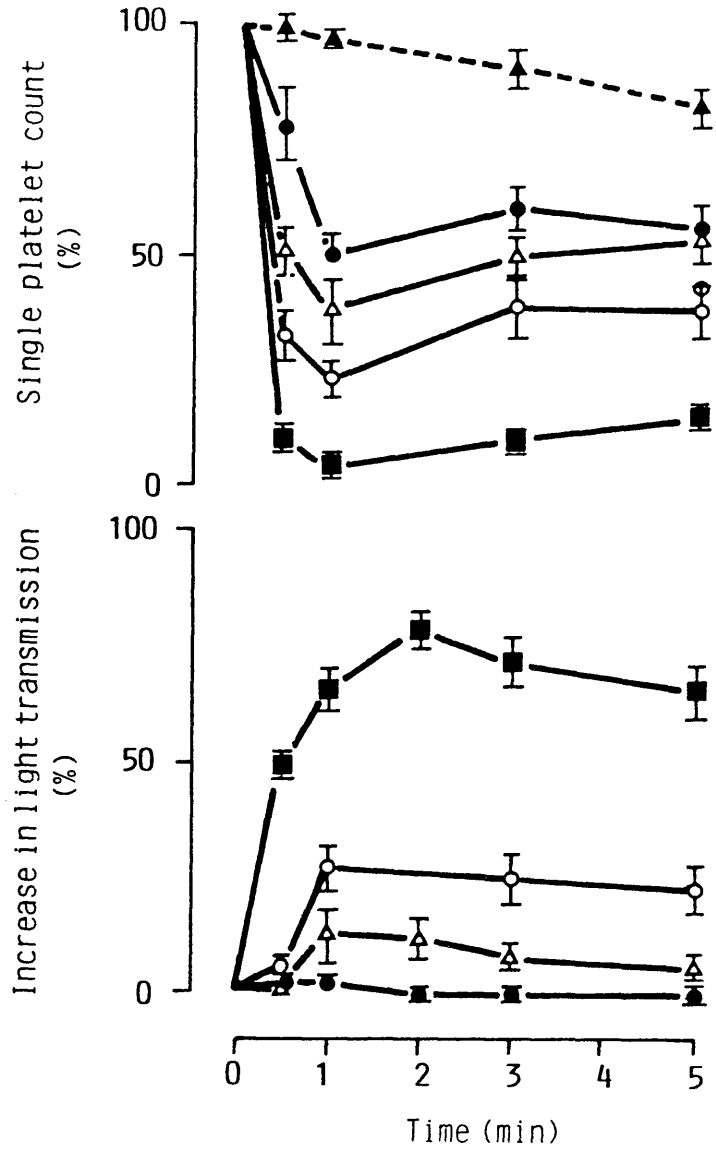

Figure 13. Platelet-rich plasma: vasopressin-induced reductions in platelet count and increases in light transmission.

Platelet-rich plasma was prepared, aggregation was monitored photometrically and samples were removed at the times indicated for the determination of platelet count as described in Methods Section. Aggregation was induced by 10 nM (●), 30 nM (△), 100 nM (o) and 300 nM (■) vasopressin or vehicle (▲). Data are the means and standard errors from three different experiments.

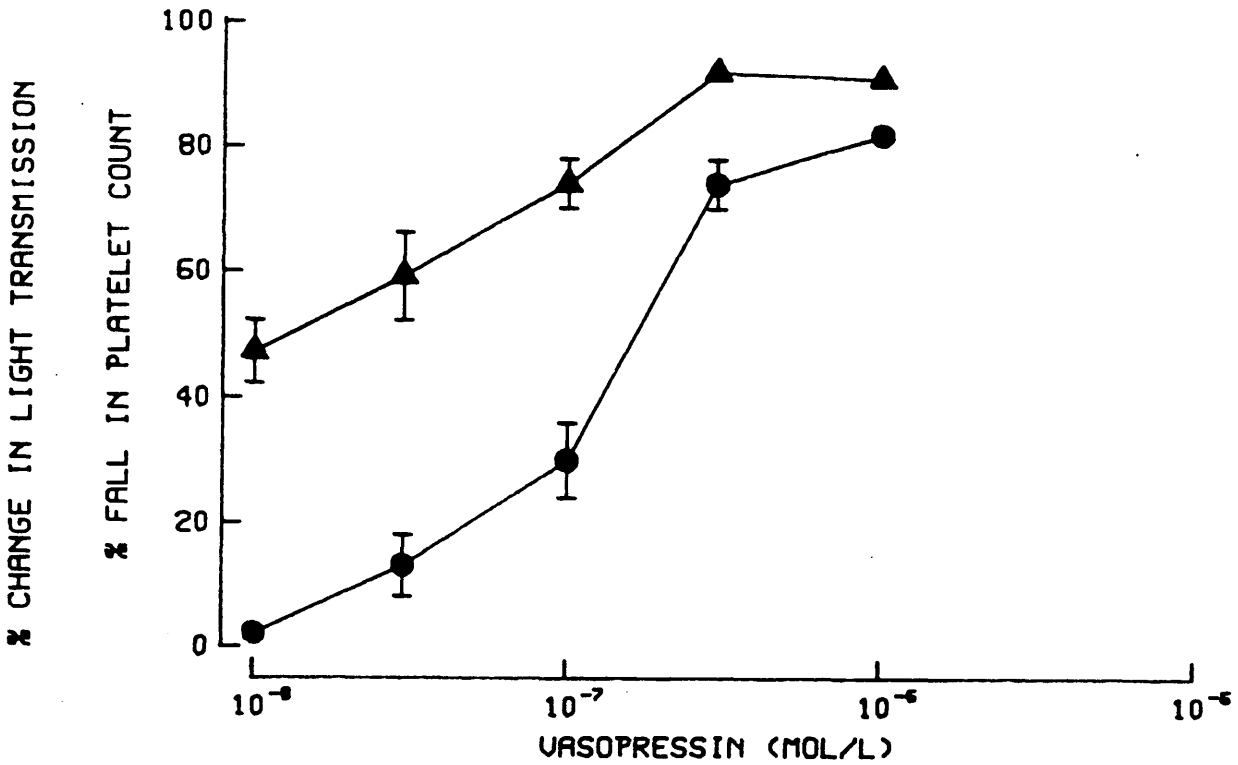

Figure 14. Platelet-rich plasma: comparison of the concentration-effect relationships for maximal vasopressin-induced increases in light transmission and reductions in platelet count.

The data show peak increases in light transmission (●) and reductions in platelet count (▲) induced by vasopressin at the concentrations indicated. Data are the means and standard errors from three different experiments.

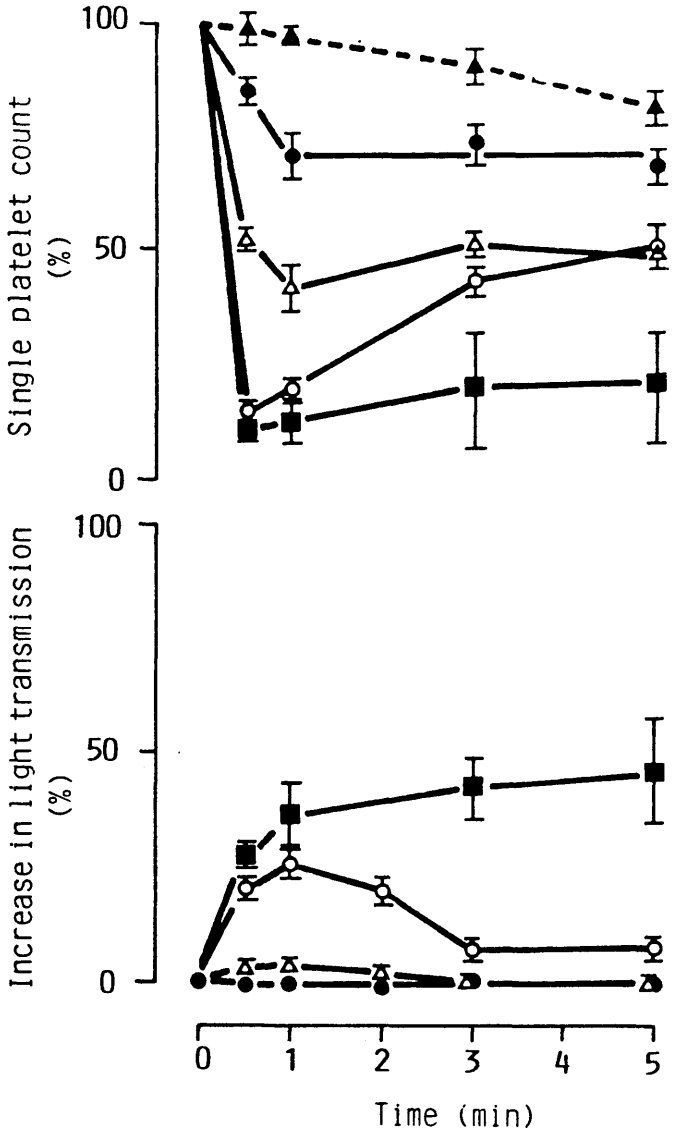

Figure 15. Platelet-rich plasma: PAF-induced reductions in platelet count and increases in light transmission.

Platelet-rich plasma was prepared, aggregation was monitored photometrically and samples were removed at the times indicated for the determination of platelet count as described in Methods Section. Aggregation was induced by 10 nM (●), 30 nM (△), 100 nM (o) and 300 nM (■) PAF or vehicle (▲). The data are means and standard errors from four different experiments.

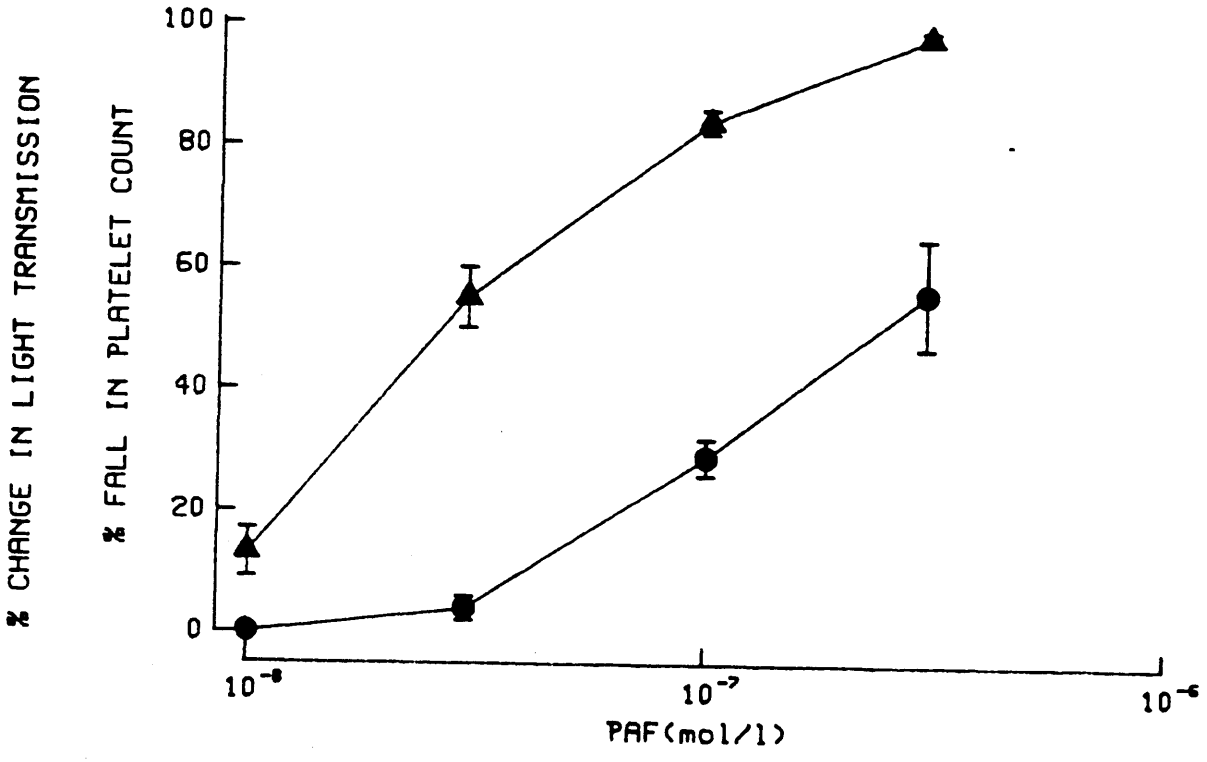

Figure 16. Platelet-rich plasma: comparison of the concentration-effect relationships for maximal PAF-induced increases in light transmission and reductions in platelet count.

The data show peak increases in light transmission (●) and reductions in platelet count (▲) induced by PAF at the concentrations shown. The data are means and standard errors from four different experiments.

($P < 0.05$). Those donors whose platelets responded poorly to vasopressin addition in whole blood (Fig. 8) failed to respond to vasopressin in PRP and were excluded from this study.

I.3. Agonist-induced platelet aggregation and ATP secretion in gel-filtered platelets

Adrenaline, ADP, vasopressin and PAF induced concentration-dependent aggregation (monitored turbidometrically) and ATP secretion from Quin 2-loaded, gel-filtered platelets. Figures 17-20 show typical aggregation and secretion traces. Responsiveness to ADP diminished rapidly (around 30 min) after preparation of gel-filtered platelets.

Having examined the platelet functional responses to agonists, I next assessed the transduction processes activated by these same agonists.

I.4. Agonist-induced changes in $[Ca^{2+}]_i$

The resting $[Ca^{2+}]_i$ in Quin 2-loaded platelets suspended in buffer A containing 1 mM Ca^{2+} was 90 $\pm$ 3 nM (mean $\pm$ S.E.M., n = 42). This was elevated in a concentration-dependent manner by ADP (0.3 - 10 μM), vasopressin (1-100 nM) and PAF (1 - 180 nM) but was not affected by adrenaline ($\leqslant$ 10 μM). Figure 21 shows typical Quin 2 fluorescence changes in response to these agonists from a single experiment. The concentration-response relationships for agonist-induced elevations in $[Ca^{2+}]_i$ are shown in Figure 22. The rank order of effectiveness in elevating $[Ca^{2+}]_i$ was PAF > vasopressin $\geqslant$ ADP > adrenaline = zero. The agonist-induced elevations in $[Ca^{2+}]_i$ were reduced by

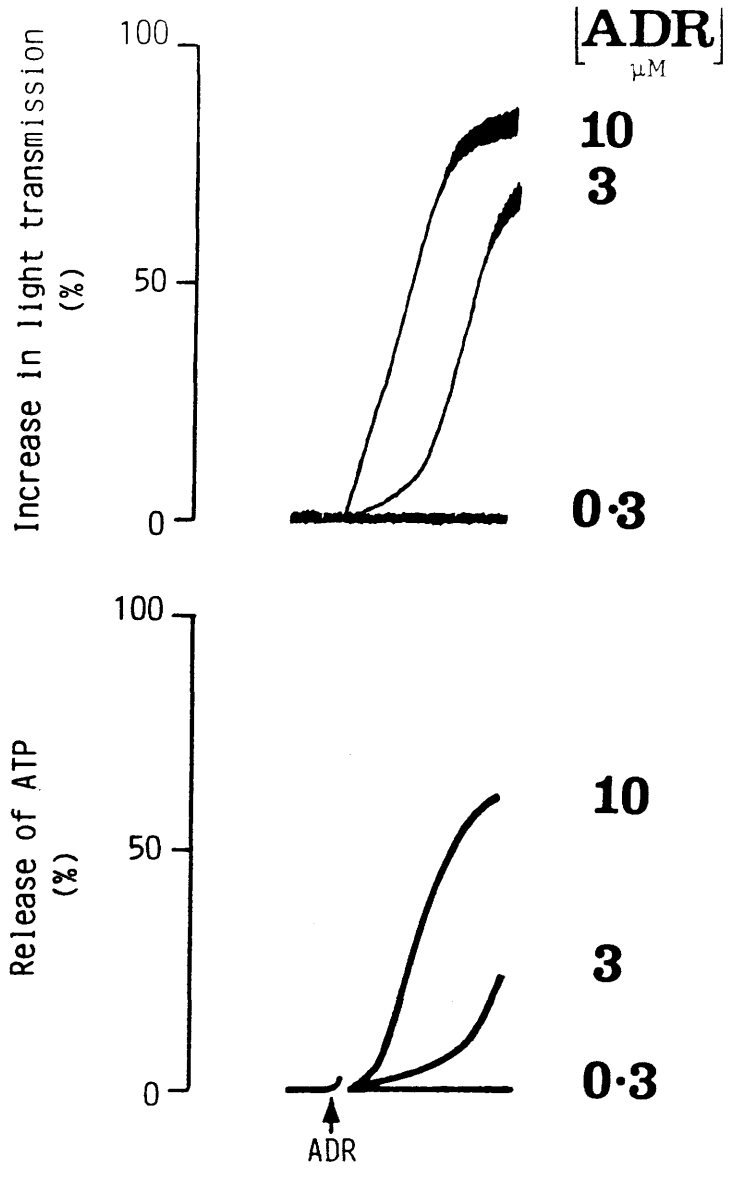

Figure 17. Aggregation and ATP secretion of Quin 2-loaded, gel-filtered platelets induced by adrenaline.

Plasma-free suspensions of Quin 2-loaded platelets were exposed to adrenaline at the concentrations indicated and aggregation and ATP secretion were monitored as described in Methods Section. The traces show typical responses from a single representative experiment.

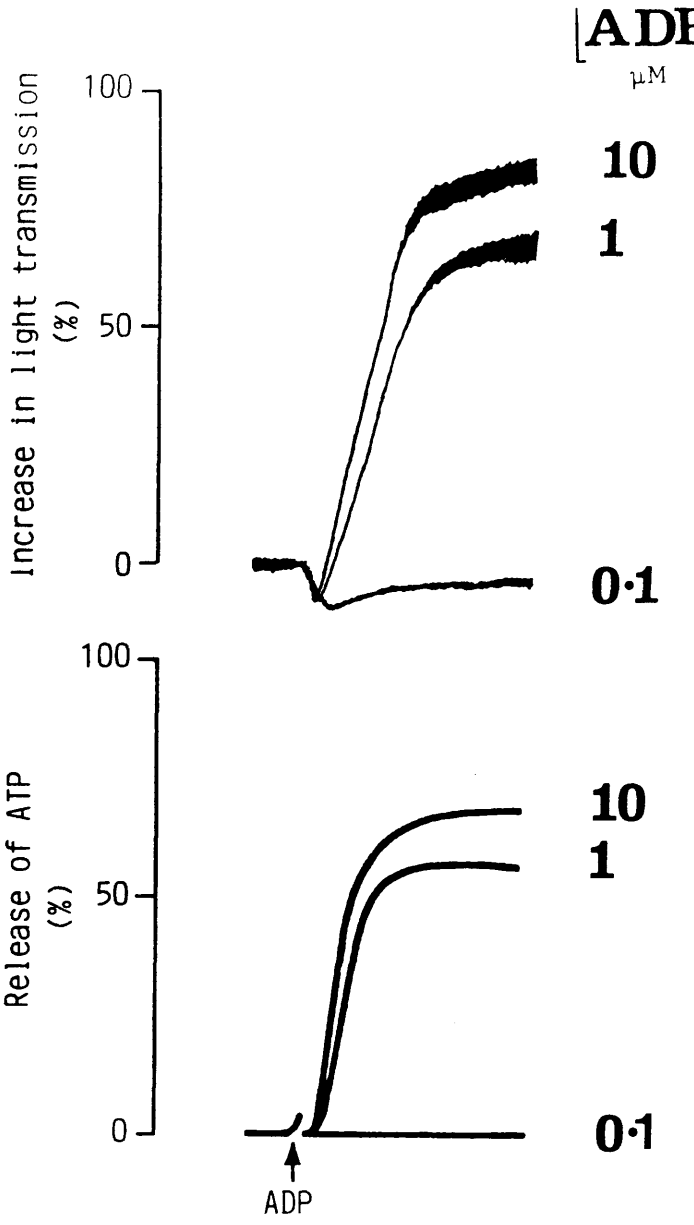

Figure 18. Aggregation and ATP secretion of Quin 2-loaded gel-filtered platelets induced by ADP.

Plasma-free suspensions of Quin 2-loaded platelets were exposed to ADP at the concentrations indicated and aggregation and ATP secretion were monitored as described in Methods Section. The traces show typical responses from a single representative experiment.

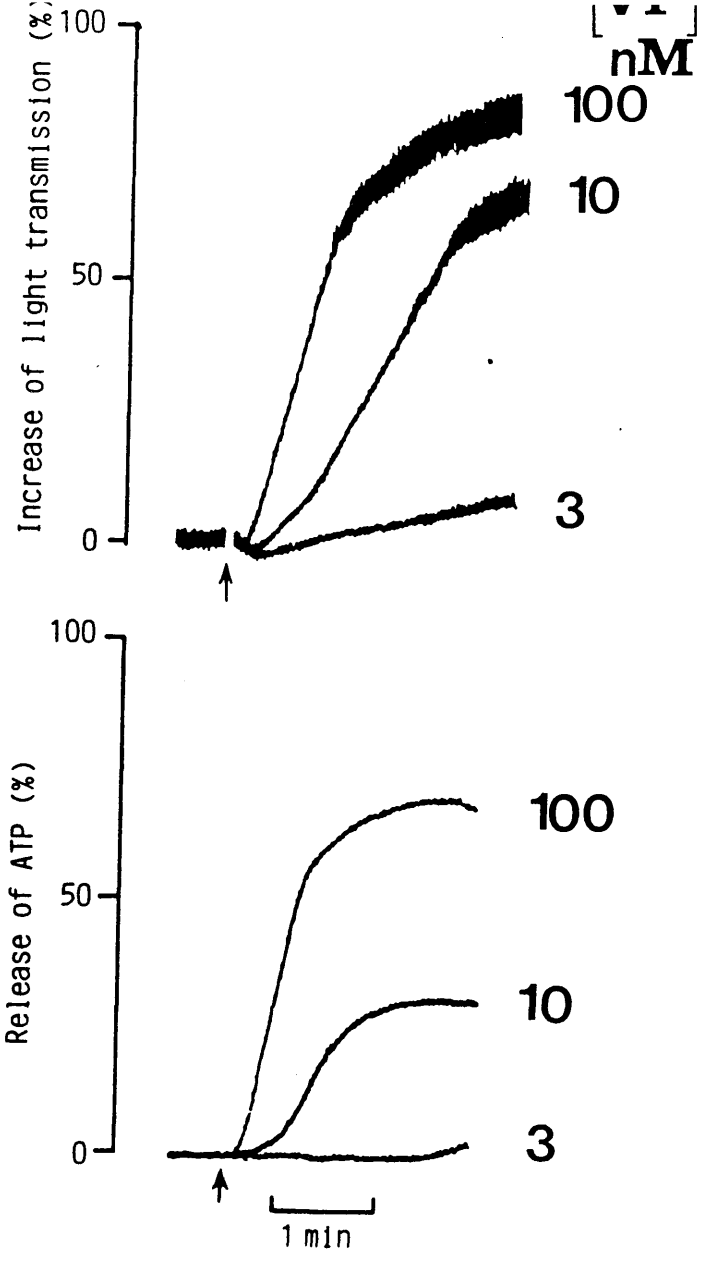

Figure 19. Aggregation and ATP secretion of Quin 2-loaded gel-filtered platelets induced by vasopressin.

Plasma-free suspensions of Quin 2-loaded platelets were exposed to vasopressin at the concentrations indicated and aggregation and ATP secretion were monitored as described in Methods Section. The traces show typical responses from a single representative experiment.

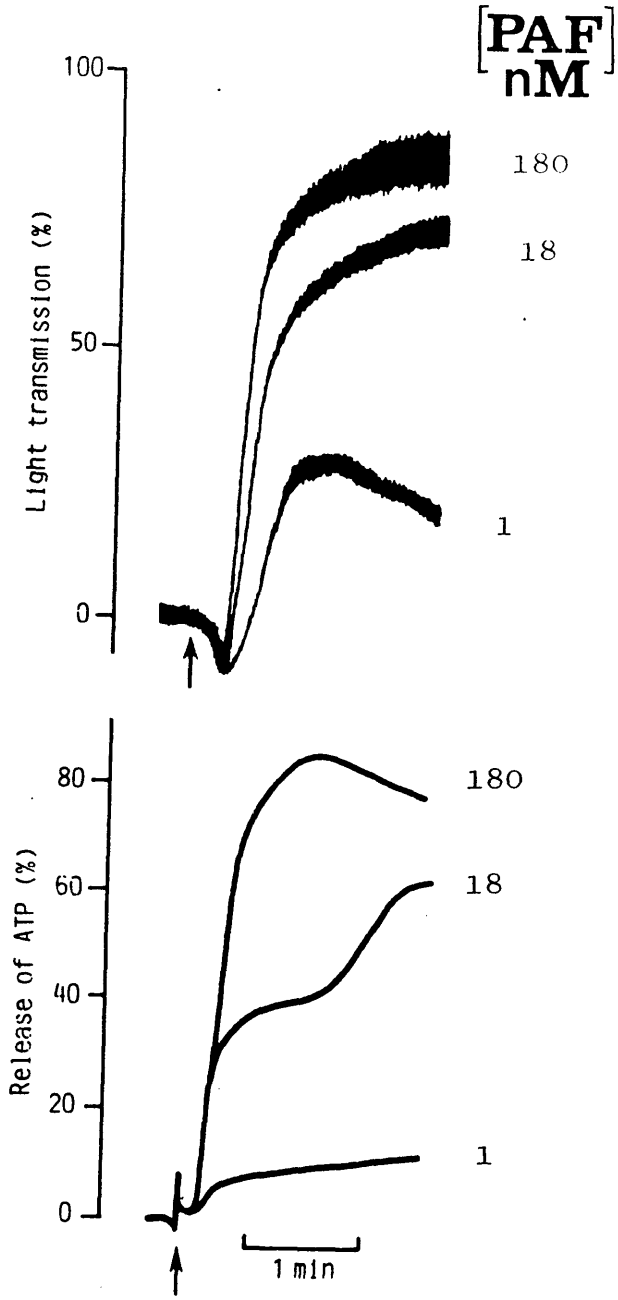

Figure 20. Aggregation and ATP secretion of Quin 2-loaded gel-filtered platelets induced by PAF.

Plasma-free suspensions of Quin 2-loaded platelets were exposed to PAF at the concentrations indicated and aggregation and ATP secretion were monitored as described in Methods Section. The traces show typical responses from a single representative experiment.

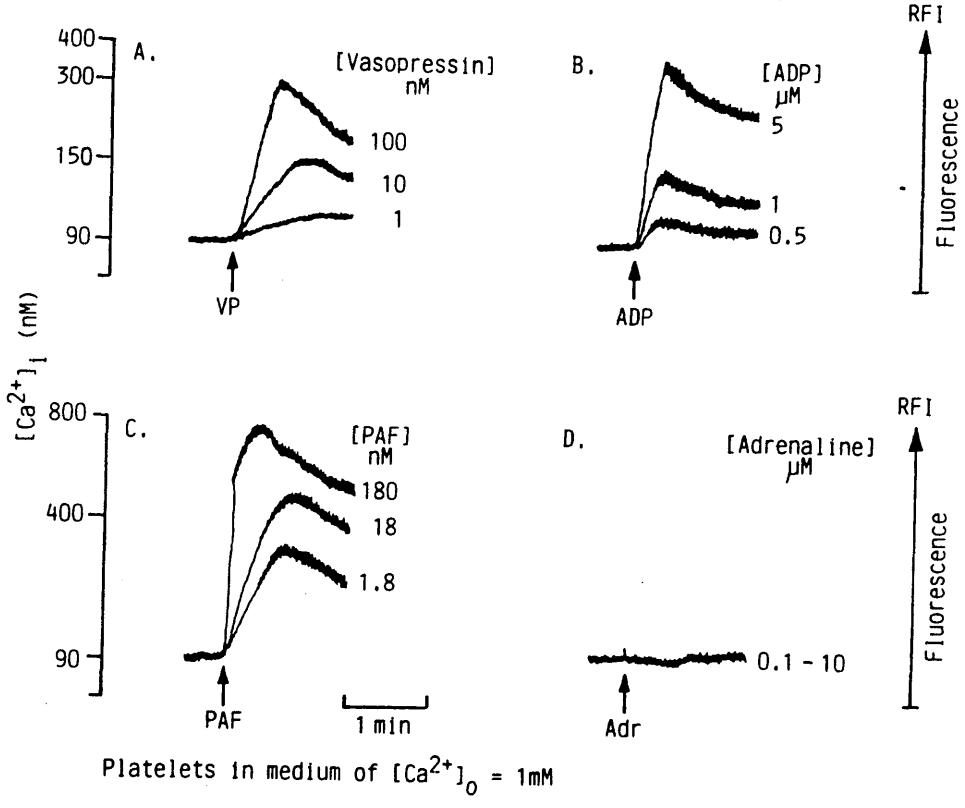

Figure 21. Effects of agonists on platelet $[Ca^{2+}]_i$.

Quin 2-loaded gel-filtered platelets were prepared and exposed to (A) vasopressin, (B) ADP, (C) PAF or (D) adrenaline at the concentrations indicated. Fluorescence was monitored and $[Ca^{2+}]_i$ was calculated as described in Methods Section. Traces show typical responses from single representative experiments.

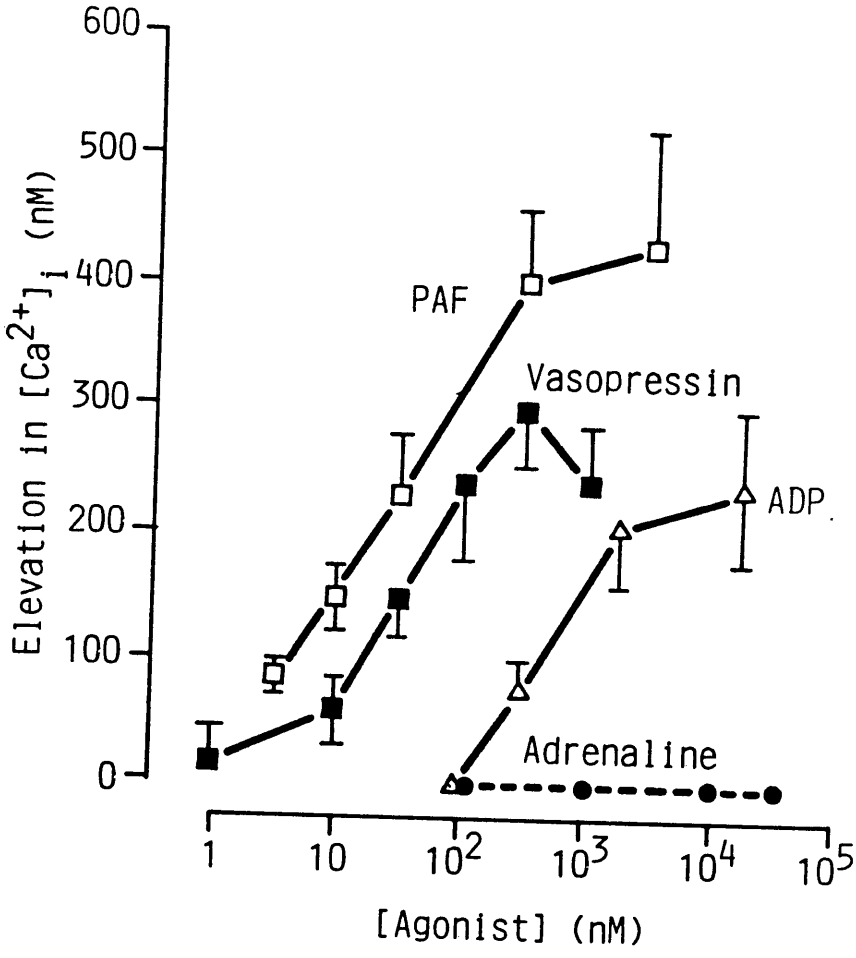

Figure 22. Concentration-response relationships for agonist-induced elevations in $[Ca^{2+}]_i$.

Agonists, at the concentrations indicated, were added to Quin 2-labelled platelets suspended in a medium $[Ca^{2+}]_o$ = 1 mM. Changes in $[Ca^{2+}]_i$ were calculated from the observed changes in dye fluorescence. Results are means and standard errors from three to nine experiments using platelets from different donors.

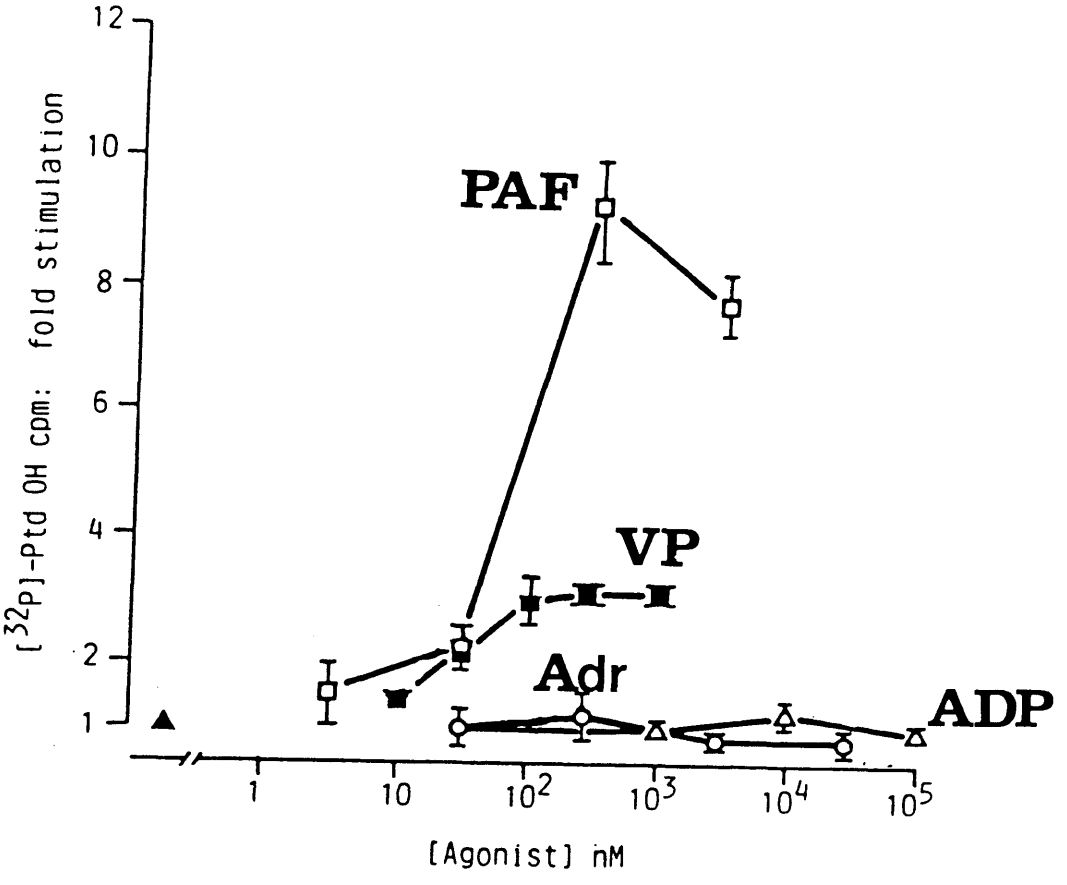

Figure 23. Agonist-induced $[^{32}P]$-Ptd OH formation in human platelets.

Samples (0.4 ml) of $[^{32}P]$-pre-labelled platelets were incubated (37°C; 60s) with agonists at the concentrations indicated and levels of $[^{32}P]$-Ptd OH were measured as described in Methods Section. To compensate for variations in the basal level of $[^{32}P]$-Ptd OH in different experiments (range = 202-608 cpm/mg protein) results are expressed as 'fold stimulation' of the basal value (=1). Data shown are representative of at least three similar experiments using platelets from different donors.

approximately 80% by incubation with EGTA (4 mM) to reduce the $[Ca^{2+}]_o$ to around 1 - 10 nM (see MacIntyre et al., 1984). This elevation of $[Ca^{2+}]_i$ in the presence of low $[Ca^{2+}]_o$ is taken to represent mobilisation of Ca^{2+} from intracellular storage sites (Hallam et al., 1984a,b; MacIntyre et al., 1985c).

I.5. Agonist-induced hydrolysis of inositol phospholipids

Using $[^{32}P]$-P_i-labelled platelets suspended in Buffer B, vasopressin (10 nM - 1 μM), and PAF (30nM - 1 μM) induced concentration dependent increases in $[^{32}P]$-Ptd OH formation. In contrast, adrenaline ($\leqslant$ 30 μM) and ADP ($\leqslant$ 100 μM) had no effect on $[^{32}P]$-Ptd OH levels in platelets (Fig. 23).

II. Platelet responses to addition of more than one stimulatory agonist

Having measured the platelet responses to addition of single agonists, I then examined the effects of agonist combinations on these responses and the accompanying changes in the transduction processes.

II.1. Effects of adrenaline on agonist-induced platelet aggregation in whole blood

(a) Effects of varying adrenaline pre-incubation time.

This study examined the effects of adding low concentrations of adrenaline simultaneously with or up to five minutes before ADP, vasopressin or PAF on the extent of agonist-induced platelet aggregation. Addition of sub-maximal concentrations of adrenaline (causing 5-15% loss of single platelets)

simultaneously with or 10, 30, 60, 120 or 300 seconds before equi-effective concentrations of ADP, vasopressin or PAF resulted in the synergistic stimulation of aggregation with rapid reductions in single platelet count of up to 80% (Figs. 24-26). The magnitude of the synergistic responses were independent of the adrenaline pre-incubation time (i.e. the maximum responses were not significantly different as judged by Student's t-test: $P > 0.1$).

(b) Concentration-response relationships.

In order to quantify the degree of potentiation of aggregation by adrenaline, the effects of pre-incubation (10 sec; 37°C) with sub- and supra-threshold concentrations of adrenaline on the agonist concentration-response relationships were determined. Adrenaline produced leftward shifts in the ADP, vasopressin and PAF concentration-response curves which were dependent upon the concentration of adrenaline used (Figs. 27A - 29A). The degree of potentiation of agonist-induced platelet aggregation was not significantly affected by incubation with aspirin (2 mM) to prevent the biosynthesis of prostaglandin endoperoxides and TxA_2 (Figs. 27B - 29B). In those cases where vasopressin failed to elicit more than 50% aggregation, adrenaline increased the maximum vasopressin-induced reduction in platelet count by 10-30% (Fig. 30A,B). These effects of adrenaline on agonist-induced platelet aggregation differed markedly from the merely additive responses seen when a low concentration of an agonist, e.g. PAF, was added 10 seconds before increasing concentrations of the same agonist (Fig. 31).

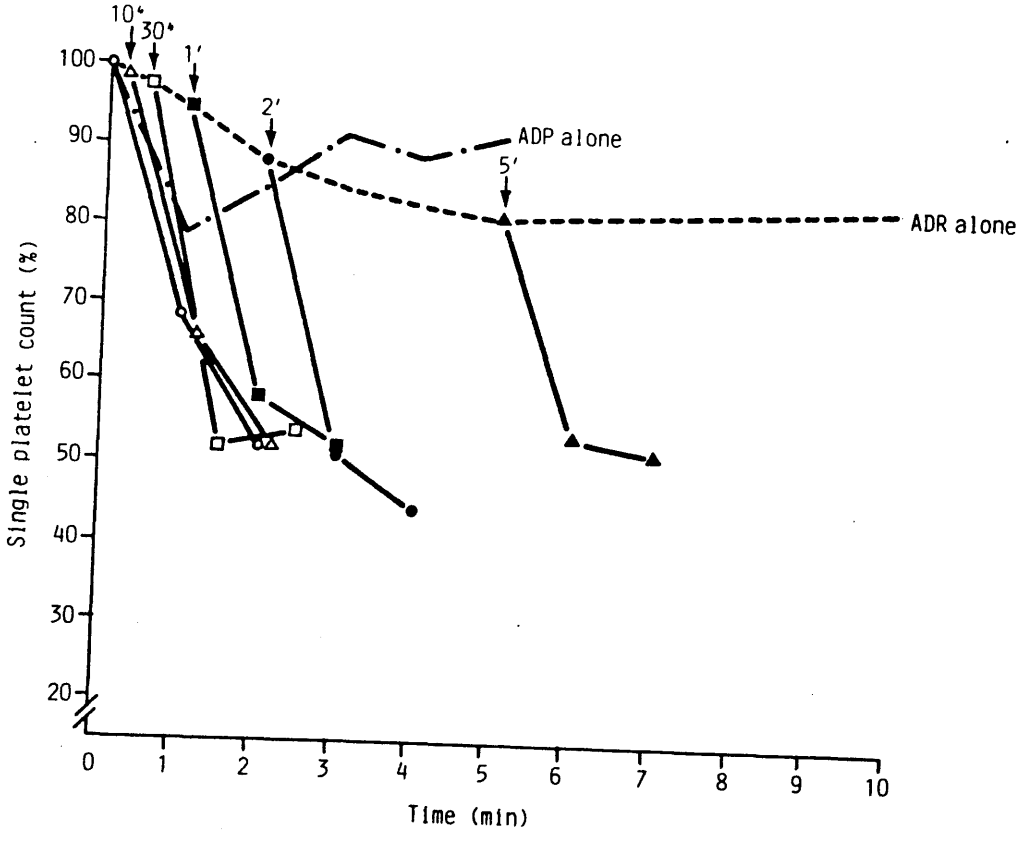

Figure 24. Effects of adrenaline on ADP-induced reductions in whole blood platelet count - effects of varying adrenaline pre-incubation time.

Platelet count was measured before and at one minute intervals after the addition of submaximal concentrations of adrenaline, 10-300 nM (---) or ADP (-.-.-.). The solid lines show the changes in platelet count after the addition of both adrenaline and ADP to the blood samples where adrenaline was added simultaneously with (o), 10 seconds (Δ), 30 seconds (□), 1 minute (■), 2 minutes (●) or 5 minutes (▲) before the ADP. The data are mean values from six different experiments. Error bars have been omitted for the sake of clarity. In each case, the peak drop in platelet count in response to addition of the combination of adrenaline and ADP was significantly greater ($P < 0.05$) than the sum of the individual responses to adrenaline and ADP added alone.

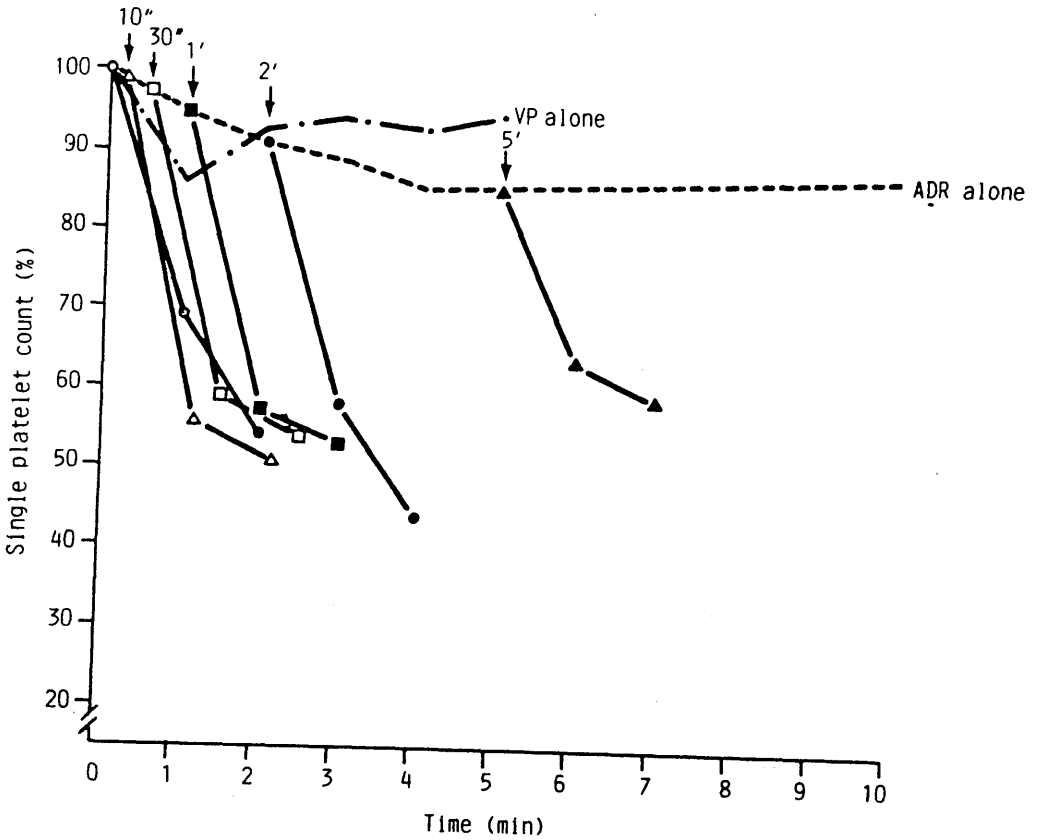

Figure 25. Effects of adrenaline on vasopressin-induced reductions in whole blood platelet count - effect of varying adrenaline pre-incubation time.

Platelet count was measured before and at one minute intervals after the addition of sub-maximal concentrations of adrenaline, 30-300 nM (---) or vasopressin, 10 nM - 1 μM (-.-.-.). The solid lines show changes in platelet count after the addition of both adrenaline and vasopressin to the blood samples where adrenaline was added simultaneously with (o), 10 seconds (Δ), 30 seconds (□), 1 minute (■), 2 minutes (●) or 5 minutes (▲) before the vasopressin. The data shows mean values from six different experiments. Error bars have been omitted for the sake of clarity. In each case the peak drop in platelet count in response to addition of the combination of adrenaline and vasopressin was significantly greater ($P < 0.05$) than the sum of the individual responses to adrenaline and vasopressin added alone.

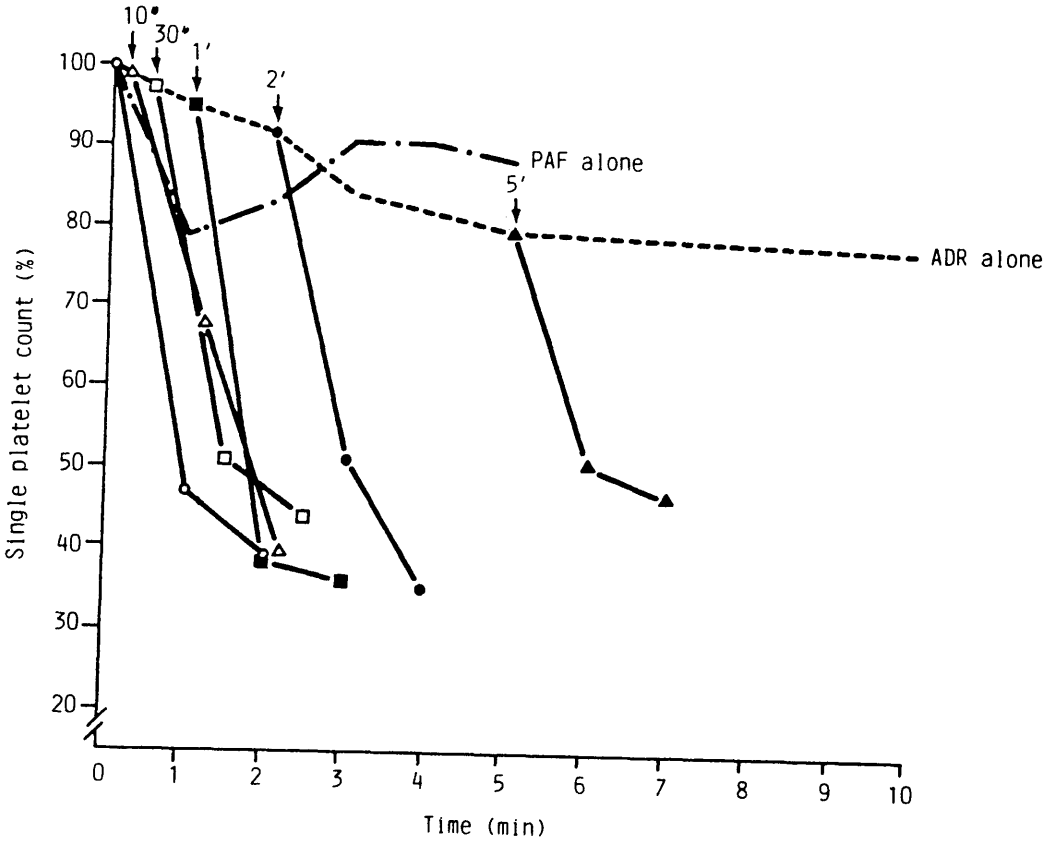

Figure 26. Effects of adrenaline on PAF-induced reductions in whole blood platelet count - effects of varying adrenaline pre-incubation time.

Platelet count was measured before and at one minute intervals after the addition of adrenaline 10-300 nm (- - -) or PAF, 10-100 nM (-.-.-.). The solid lines show changes in platelet count after the addition of both adrenaline and PAF to the blood samples where adrenaline was added simultaneously with (o), 10 seconds (Δ), 30 seconds (□), 1 minute (■), 2 minutes (●) or 5 minutes (▲) before the PAF. The data shows mean values from six different experiments. Error bars have been omitted for the sake of clarity. In each case the peak drop in platelet count in response to addition of the combination of adrenaline and PAF was significantly greater ($P < 0.05$) than the sum of the individual responses to adrenaline or PAF added alone.

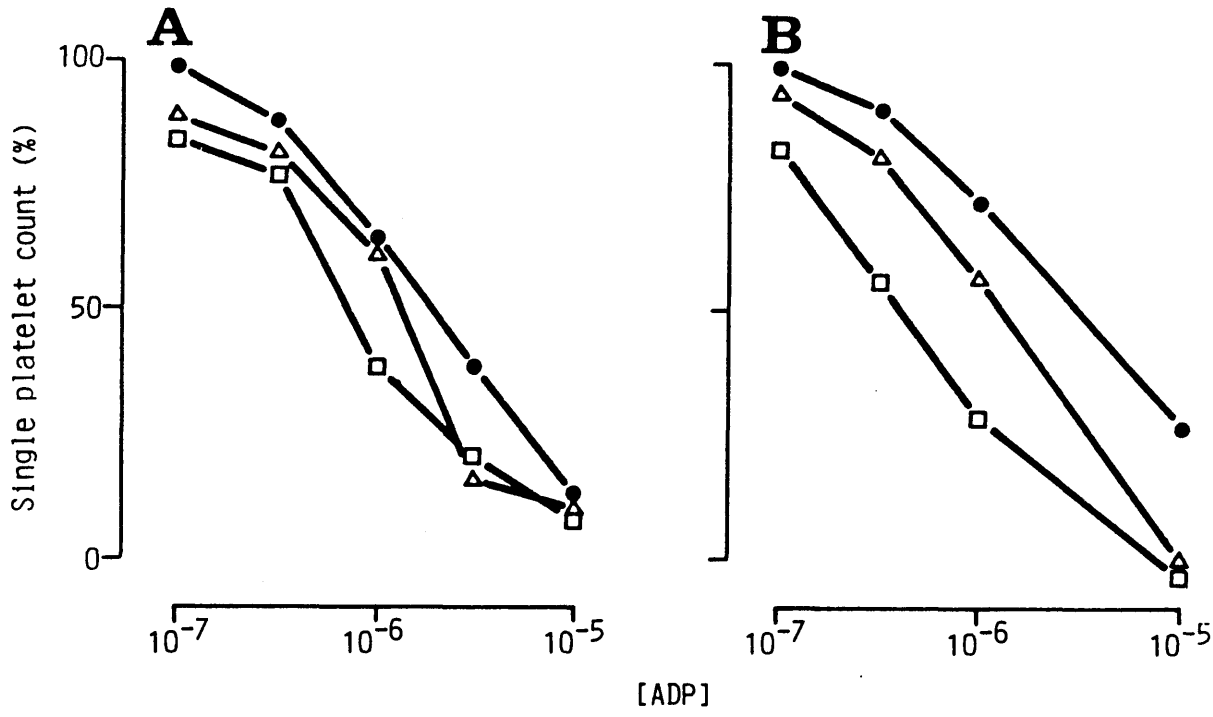

Figure 27. Effects of adrenaline on the concentration-effect relationships for reduction in whole blood platelet count induced by ADP.

The maximum reduction in whole blood platelet count was monitored in response to ADP (●); ADP plus a sub-threshold concentration of adrenaline, 30 nM (△) or ADP plus a concentration of adrenaline giving 5-10% reduction in platelet count when added alone, 100 nM (□) in the absence (A) or presence (B) of aspirin (2 mM). Adrenaline, where present, was added 10 seconds before the ADP. The data are from a single experiment representative of five.

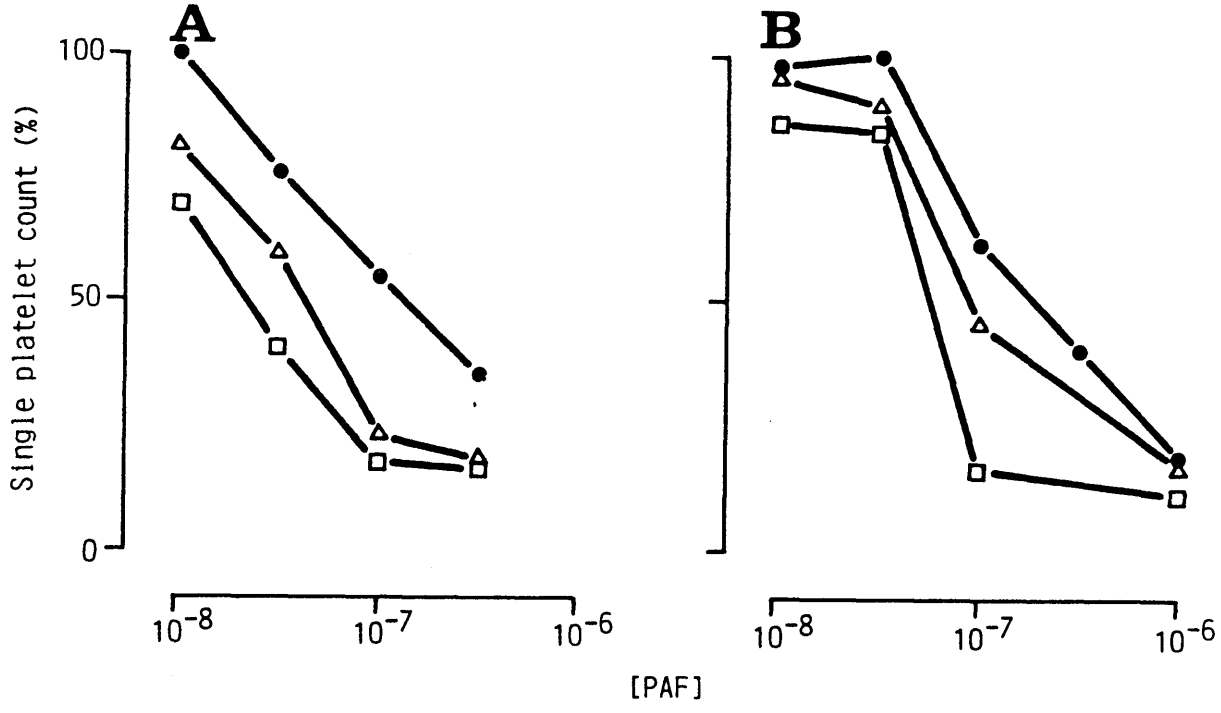

Figure 28. Effects of adrenaline on the concentration-effect relationships for reduction in whole blood platelet count induced by PAF.

The maximum reduction in whole blood platelet count was monitored in response to PAF (●); PAF plus a sub-threshold concentration of adrenaline, 100 nM (△) or PAF plus a concentration of adrenaline giving 5-10% reduction in platelet count when added alone, 300 nM (□) in the absence (A) and presence (B) of aspirin (2 mM). Adrenaline, when present, was added 10 seconds before the PAF. The data are from a single experiment representative of five.

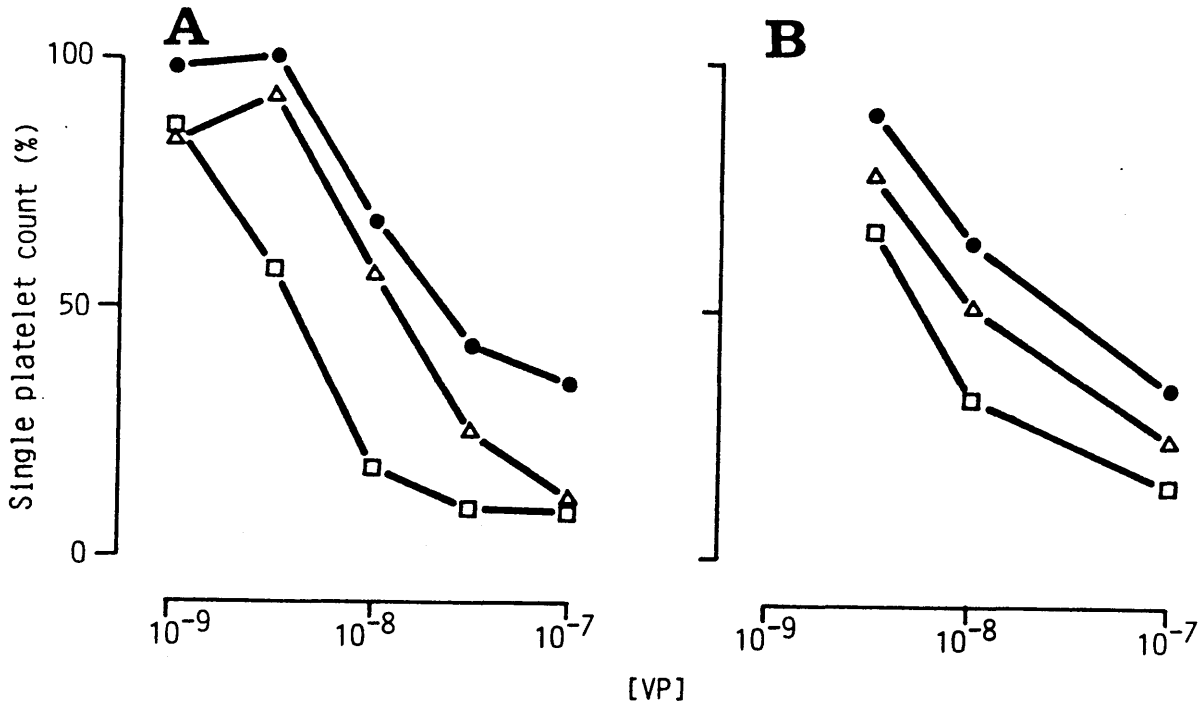

Figure 29. Effects of adrenaline on the concentration-effect relationships for reduction in whole blood platelet count induced by vasopressin.

The maximum reduction in whole blood platelet count was monitored in response to vasopressin (●); vasopressin plus a sub-threshold concentration of adrenaline, 30 nM (△) or vasopressin plus a concentration of adrenaline giving 5-10% reduction in platelet count when added alone, 100 nM (□) in the absence (A) or presence (B) of aspirin (2 mM). The data are from a single experiment representative of three.

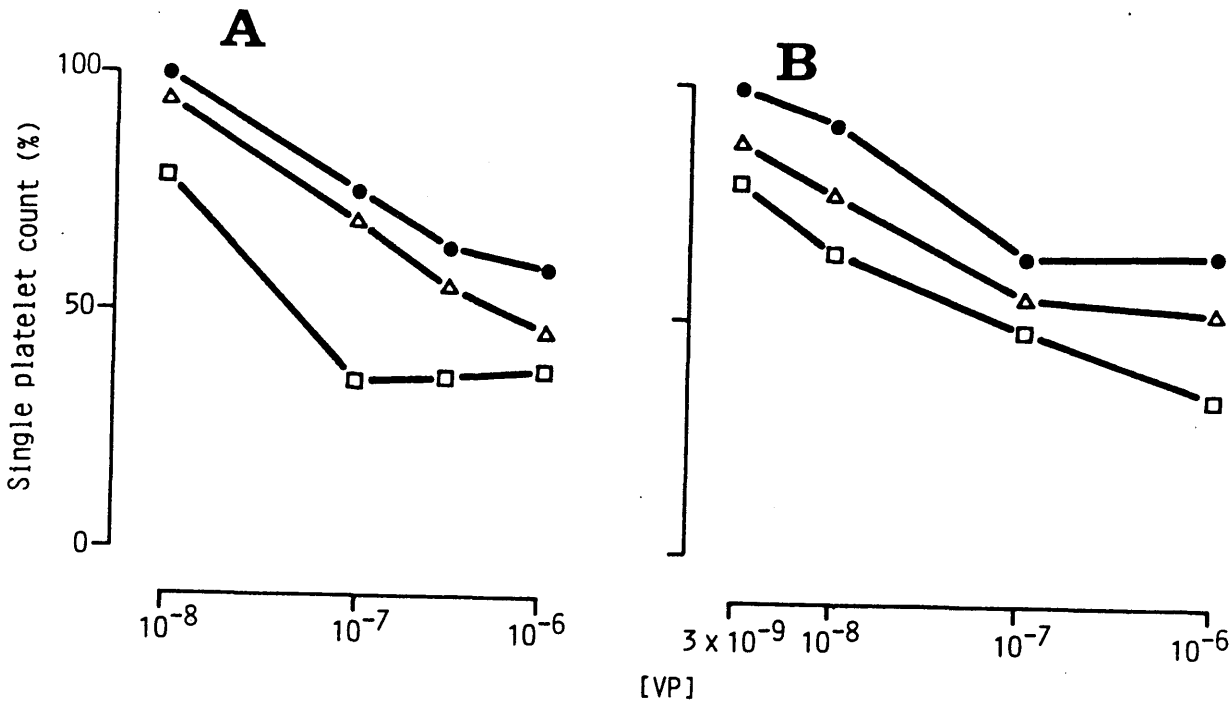

Figure 30. Effects of adrenaline on the concentration-effect relationships for reduction in whole blood platelet count induced by vasopressin: poor responses seen in some individuals.

The maximum reduction in whole blood platelet count was monitored in response to vasopressin (●); vasopressin plus a sub-threshold concentration of adrenaline, 30 nM (Δ) or vasopressin plus a concentration of adrenaline giving 5-10% reduction in platelet count when added alone (□) in the absence (A) and presence (B) of aspirin (2 mM). The data are from a single experiment representative of four.

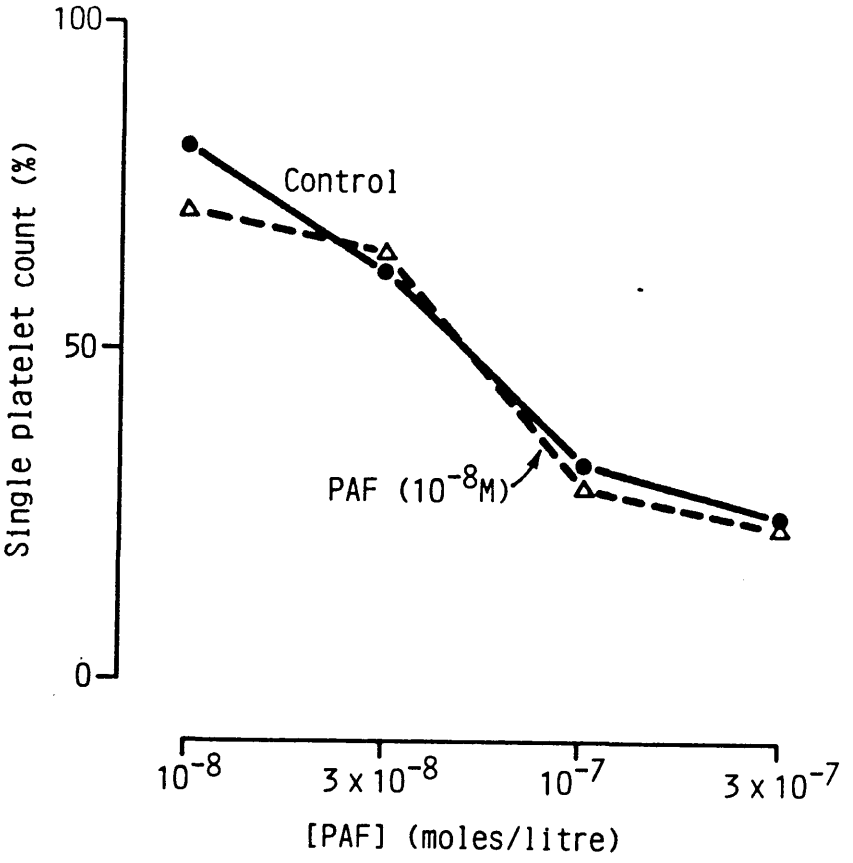

Figure 31. Effects of pre-addition of a sub-maximal concentration of PAF of the concentration-effect relationship for reduction in whole blood platelet count induced by PAF.

The maximum PAF-induced reduction in whole blood platelet count was monitored in control samples (●) and in samples pre-incubated (10 sec) with PAF, 10 nM (△). The data are from a single experiment representative of four.

II.2. Effects of adrenaline on agonist-induced activation of Quin 2-loaded gel-filtered platelets

This study examined the effects of subthreshold concentrations of adrenaline on aggregation and ATP secretion induced by ADP, vasopressin and PAF in Quin 2-loaded gel-filtered platelets. In agreement with previous reports using PRP (Ardlie et al., 1966; Grant & Scrutton, 1980a; Vargaftig et al., 1982), aggregation induced by sub-maximal concentrations of ADP (0.3 - 3 μM), vasopressin (3 - 30 nM) and PAF (1 - 18 nM) were augmented significantly by simultaneous or prior (10 - 60 sec) addition of sub-threshold concentrations of adrenaline (0.1 - 1 μM). The degree of potentiation observed was independent of the adrenaline pre-incubation time (Figs. 32A - 34). Adrenaline (0.1 - 1 μM) also potentiated agonist-induced ATP secretion (shown in Figure 32B for vasopressin).

The mechanisms underlying potentiation of platelet activation by adrenaline were assessed by examining the effects of (a) adrenaline on agonist-induced elevation of $[Ca^{2+}]_i$; (b) adrenaline on agonist-induced $[^{32}P]$-Ptd OH formation and (c) activation of protein kinase C and elevation of $[Ca^{2+}]_i$ alone and in combination on agonist-induced platelet activation.

II.3. The roles of $[Ca^{2+}]_i$ and inositol phospholipid hydrolysis in the potentiation of platelet activation by adrenaline

(a) Effects of adrenaline on agonist-induced elevation of $[Ca^{2+}]_i$.

$[Ca^{2+}]_i$ was monitored in parallel with the aggregation and secretion responses described above (Section II.2). Addition of adrenaline (0.1 - 1 μM) simultaneously with or up to 60 sec

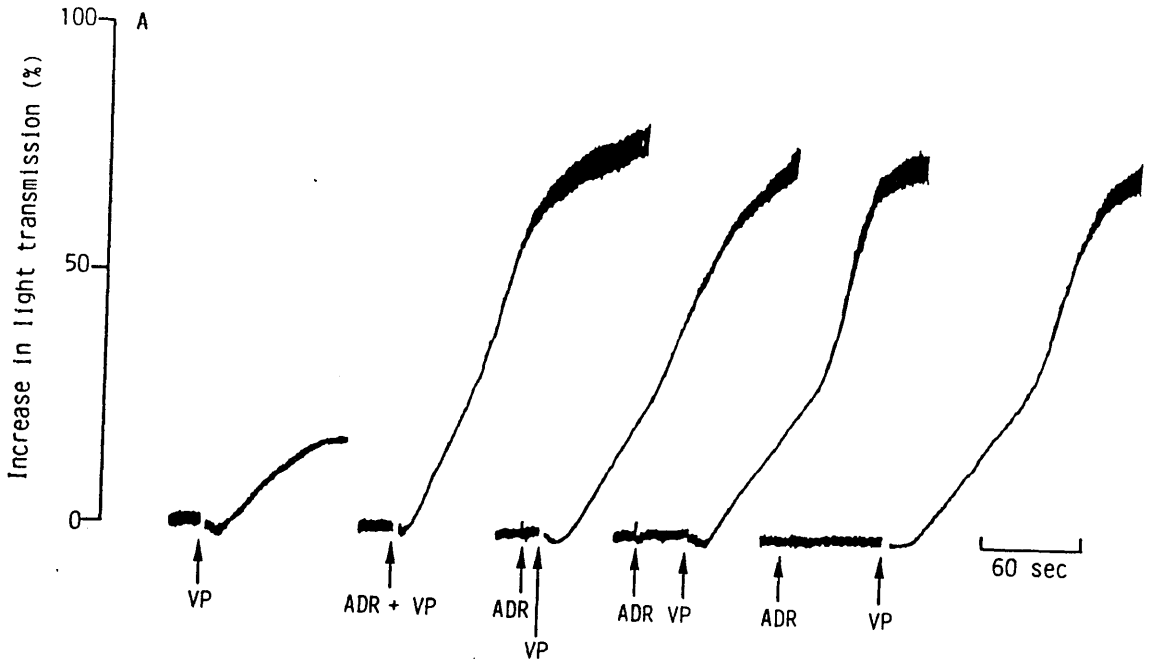

Figure 32(A). Effects of adrenaline on vasopressin-induced aggregation of Quin 2-loaded gel-filtered platelets - typical aggregometer traces.

Vasopressin, VP (3 nM) was added to stirred samples of Quin 2-loaded platelets which had been incubated with saline or adrenaline, ADR (100 nM) for the times indicated and aggregation was monitored photometrically as described in Methods Section. Prolonged incubation with adrenaline (100 nM) did not result in any increase in light transmission and inclusion of the cyclo-oxygenase inhibitor flurbiprofen (10 μM) in the incubation medium did not affect the pattern of responsiveness.

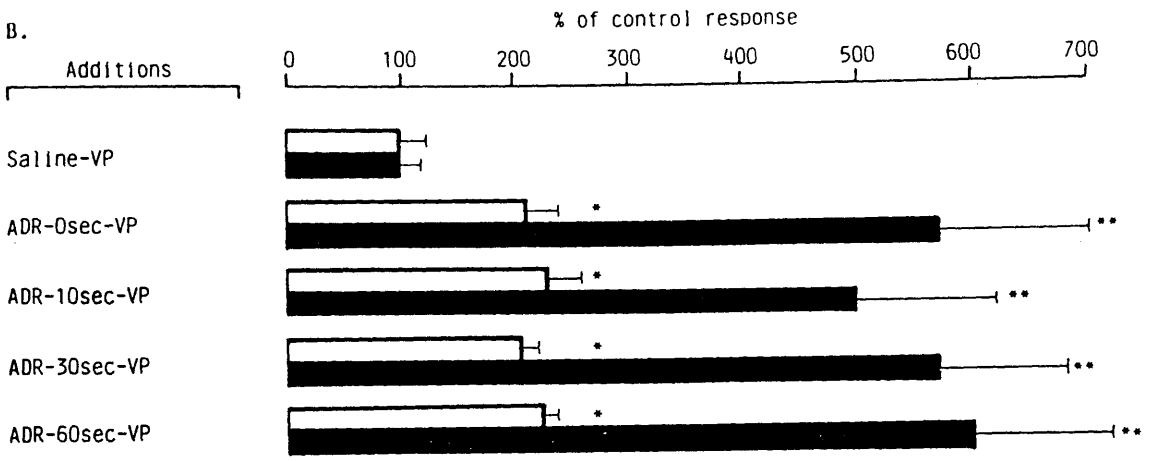

Figure 32(B). Effects of adrenaline on vasopressin-induced aggregation and ATP secretion of Quin 2-loaded gel-filtered platelets - bar graphs.

Vasopressin was added to stirred samples of Quin 2-loaded gel-filtered platelets which had been incubated with saline or adrenaline (0.1 μM) for the times indicated and the initial rate of aggregation (open bars) and the degree of ATP secretion (solid bars) were determined as described in Methods Section. To facilitate comparison between different experiments, results are expressed as percentage of the control response to vasopressin plus saline in each experiment. The data are means and standard errors from three different experiments. Data were compared to saline controls using Student's t-test: *$P < 0.05$; ** $P < 0.01$.

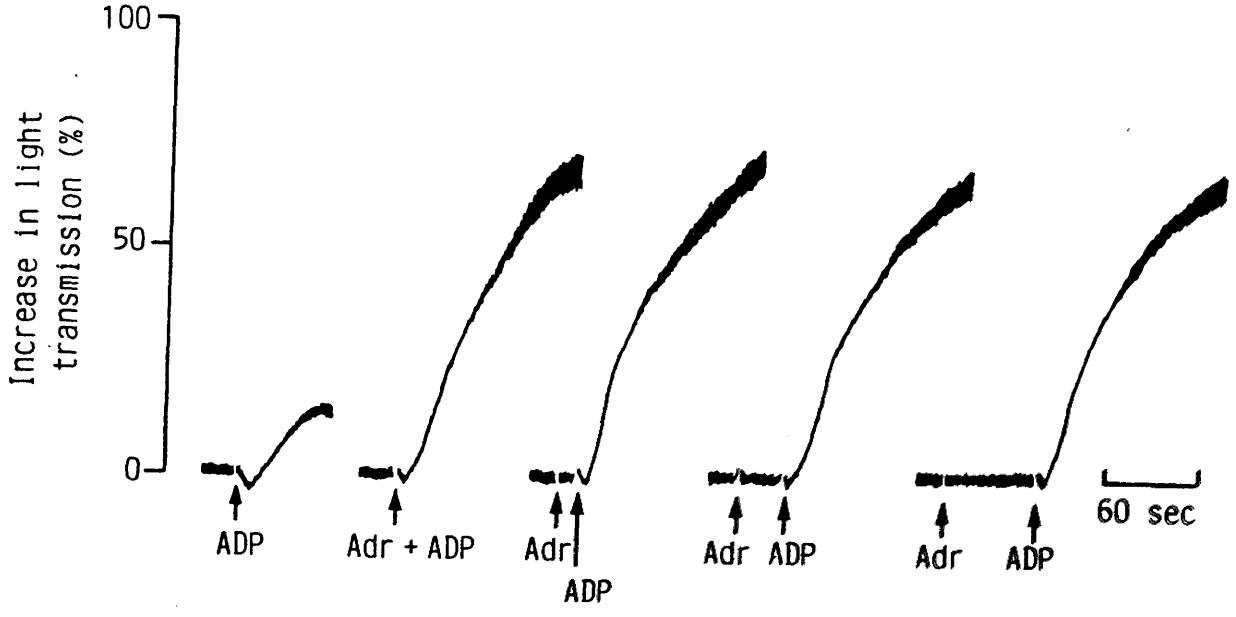

Figure 33. Effects of adrenaline on ADP-induced aggregation of Quin 2-loaded gel-filtered platelets.

ADP (300 nM) was added to stirred samples of Quin 2-loaded platelets which had been incubated with adrenaline, Adr (100 nM) for the times indicated and aggregation was monitored photometrically as described in Methods Section. Traces show typical aggregation responses from a single representative experiment.

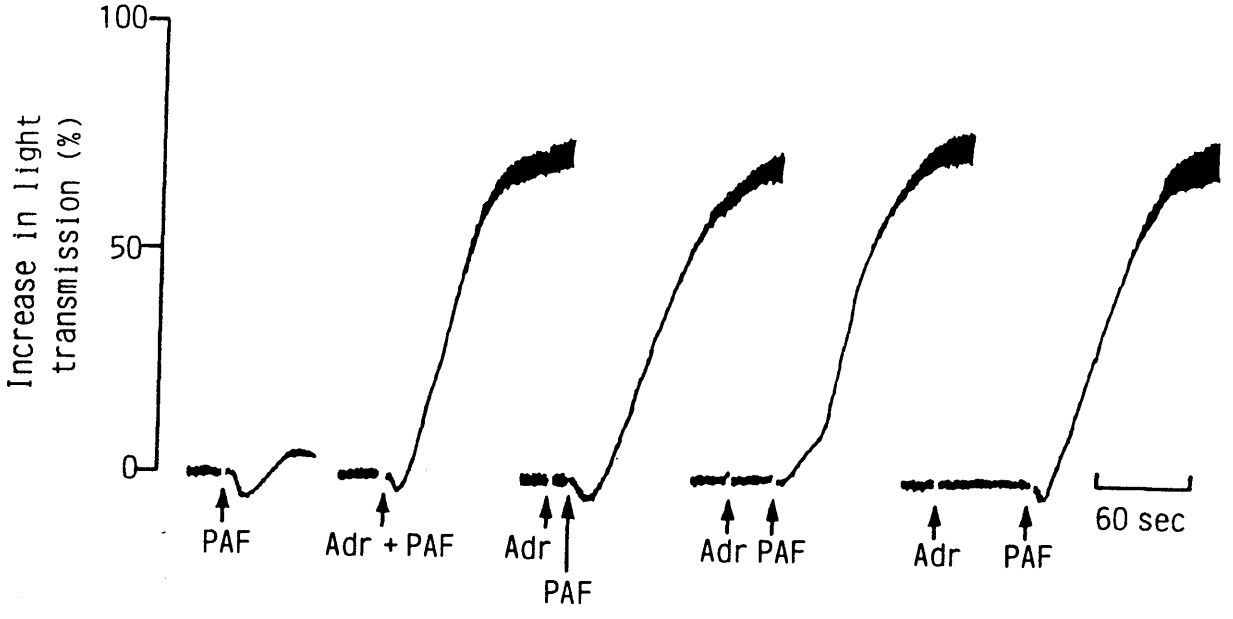

Figure 34. Effects of adrenaline on PAF-induced aggregation of Quin 2-loaded gel-filtered platelets.

PAF (3 nM) was added to stirred samples of Quin 2-loaded platelets which had been incubated with adrenaline, ADR (100 nM) for the times indicated and aggregation was monitored photometrically as described in Methods Section. Traces show typical aggregation responses from a single representative experiment.

before sub-maximal concentrations of ADP (1 μM), vasopressin (3 - 10 nM) or PAF (1 - 18 nM) resulted in enhancement of agonist-induced elevations in $[Ca^{2+}]_i$ (Figs. 35A-C). The degree of potentiation of $[Ca^{2+}]_i$ was apparently dependent upon the adrenaline pre-incubation time. Potentiation of vasopressin-induced elevation of $[Ca^{2+}]_i$ by adrenaline (10 sec pre-incubation) was not significantly affected by incubation (30-60 min) with the cyclo-oxygenase inhibitor flurbiprofen, 10 μM (Fig. 36). Vasopressin (10 nM)-induced mobilisation of internal Ca^{2+} (i.e. the $[Ca^{2+}]_i$ rise monitored in the presence of EGTA, 4 mM) also was potentiated by 60 ± 17% (mean ± S.E.M., n = 3) following pre-incubation (10 sec) with adrenaline (0.1 μM). It should be noted that, in all cases, the magnitude of the elevations in $[Ca^{2+}]_i$ were relatively small, even in the presence of adrenaline where maximal aggregation and ATP secretion occurred. The rises elicited by the various agonists in the presence of 1 mM $[Ca^{2+}]_o$ are listed in Table 3.

(b) Effects of adrenaline on vasopressin-induced inositol phospholipid hydrolysis.

To investigate the possible involvement of inositol phospholipid hydrolysis and DG formation in the potentiation of platelet activation, the effects of adrenaline on vasopressin-induced $[^{32}P]$-Ptd OH formation were examined. The stimulation of $[^{32}P]$-Ptd OH formation by sub-maximal concentrations of vasopressin (10 - 100 nM) was augmented by adrenaline: significant potentiation was observed when adrenaline was added simultaneously with or 10-60 sec before vasopressin (Fig. 37).

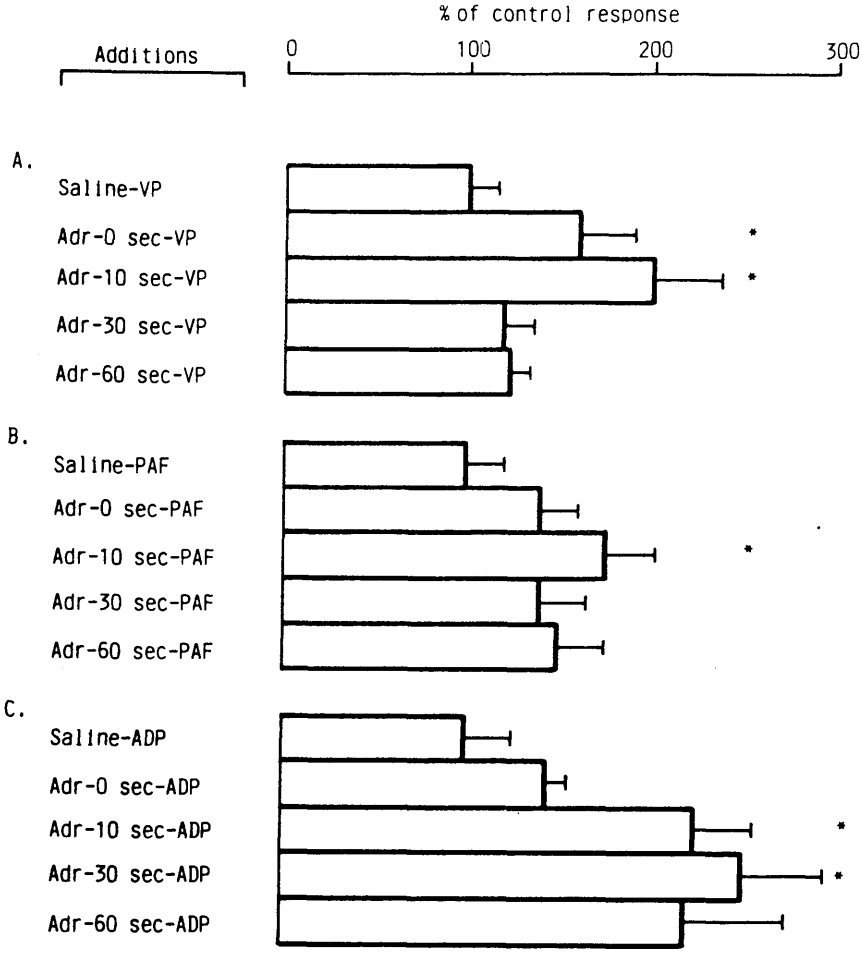

Figure 35. Effects of adrenaline on agonist-induced elevation of $[Ca^{2+}]_i$.

Quin 2-loaded gel-filtered platelets, prepared as described in Methods Section, were incubated with saline or adrenaline (1 μM) for the times indicated prior to the addition of (A) vasopressin (3-10 nM); (B) PAF (1-18 nM) or (C) ADP (0.3 - 1 μM). Fluorescence was monitored and Ca^{2+}_i was calculated as described in Methods Section. The data are means and standard errors from three to seven experiments and are expressed as percentage of the control (saline-treated) elevation of $[Ca^{2+}]_i$ above the resting level. Basal $[Ca^{2+}]_i$ was 90 ± 3 nM and the control (100%) elevations in $[Ca^{2+}]_i$ were (A) VP: 80 ± 8 nM; (B) PAF: 70 ± 12 nM and (C) ADP: 35 ± 9 nM. Data were compared to saline controls using Student's t-test: *$P < 0.05$.

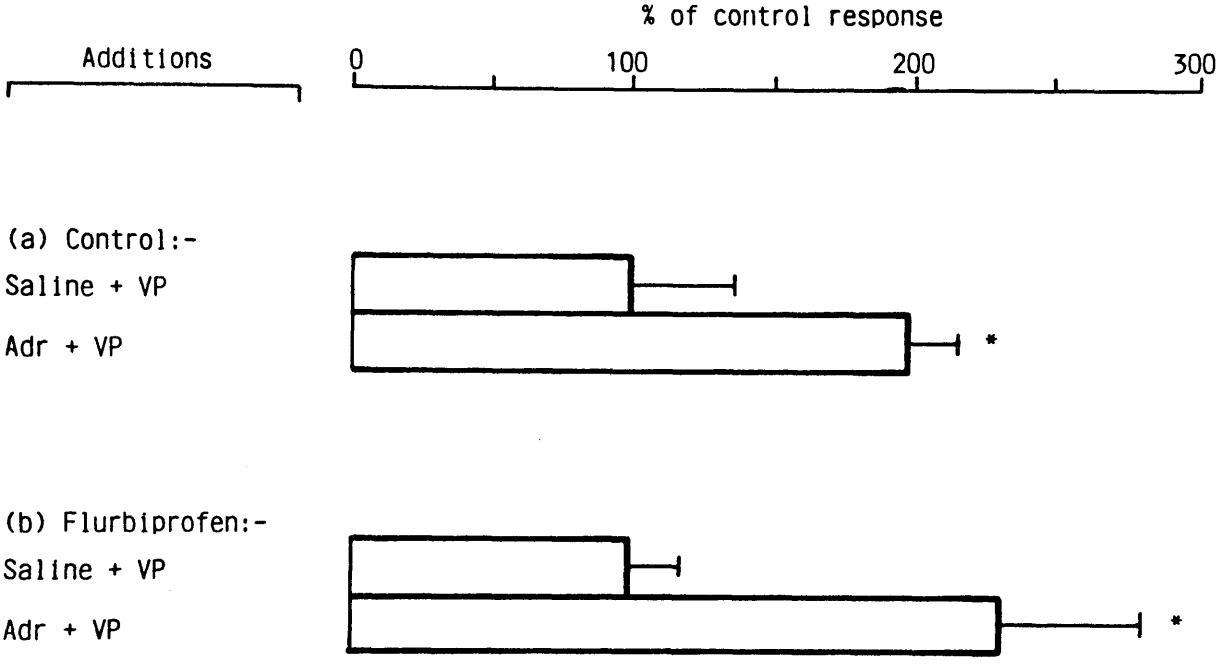

Figure 36. Effects of flurbiprofen on potentiation of vasopressin-induced elevation of $[Ca^{2+}]_i$ by adrenaline.

Quin 2-loaded gel-filtered platelets prepared as described in Methods Section were incubated with saline or adrenaline (100 nM) for 10 seconds prior to the addition of vasopressin (10 nM) in the absence (a) or presence (b) of flurbiprofen (10 μM; 30 min incubation). Elevations in $[Ca^{2+}]_i$ above the resting level (90 $\pm$ 3 nM) were monitored as described in Methods Section. The control rises in $[Ca^{2+}]_i$ were (a) 45 $\pm$ 15 nM and (b) 30 $\pm$ 5 nM. Data are from a single experiment (representative of three similar) and were compared to saline controls using Student's t-test: *$P < 0.05$.

TABLE 3. Effects of adrenaline on agonist-induced elevations in $[Ca^{2+}]_i$.

Agonist	Elevation in $[Ca^{2+}]_i$ (nM)				
		Adrenaline preincubation time			
	Control	0"	10"	30"	60"
ADP (1 μM)	35 ± 5	50 ± 4	77 ± 11	85 ± 14	74 ± 20
VP (10 nM)	80 ± 8	125 ± 30	160 ± 25	95 ± 15	100 ± 10
PAF (1 nM)	75 ± 9	105 ± 3	130 ± 20	105 ± 15	115 ± 15

Samples (2 ml) were incubated (37°C) with saline (vehicle) or adrenaline (0.1 - 1 μM) for the times indicated prior to the addition of ADP (1 μM), vasopressin (10 nM) or PAF (1 nM) and increases in $[Ca^{2+}]_i$ were monitored as described in Methods Section. The data are means and standard errors from three to seven experiments using blood from different donors.

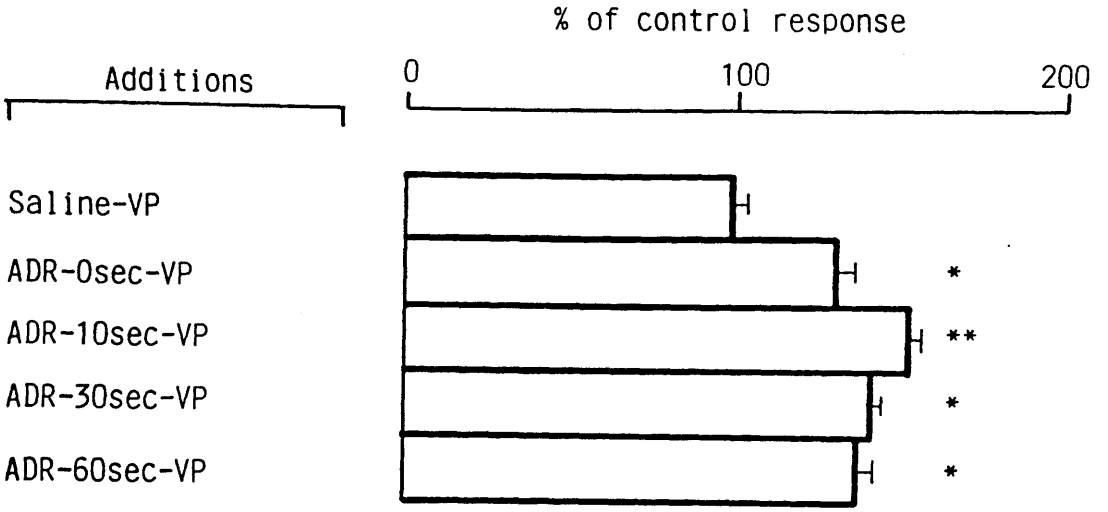

Figure 37. Effects of adrenaline on vasopressin-induced $[^{32}P]$-Ptd OH formation.

Plasma-free suspensions of $[^{32}P]$-P_i labelled platelets, prepared as described in Methods Section, were incubated with saline or adrenaline, ADR (100 nM) for the times indicated prior to the addition of vasopressin, VP (30 nM) for a further 30 seconds. $[^{32}P]$-Ptd OH formation was monitored as described in Methods Section. Incubations were carried out in the presence of flurbiprofen (10 μM). Results are expressed as percentage of the saline control which represents a 2-3 fold stimulation of $[^{32}P]$-Ptd OH formation. Data are means and standard errors from a single experiment which was typical of three similar experiments. Data were compared to saline control using Student's t-test: $*P < 0.05$; $**P < 0.005$.

(c) Effects of ionomycin on $[Ca^{2+}]_i$ and vasopressin-induced platelet activation.

To clarify the role of elevation of $[Ca^{2+}]_i$ in potentiation of platelet activation, the effects of the Ca^{2+}-ionophore, ionomycin on $[Ca^{2+}]_i$ and on aggregation and ATP secretion induced by sub-maximal concentrations of vasopressin were examined. In these studies, due to the strong affinity of ionomycin for albumin, bovine serum albumin was omitted from the buffer (Buffer A). Under these conditions, approximately ten-fold lower concentrations of vasopressin were required to induce platelet activation. Ionomycin (10 - 100 nM) evoked elevations in $[Ca^{2+}]_i$ which were approximately additive with vasopressin (1 nM)-induced rises in $[Ca^{2+}]_i$ when the ionomycin was added 10 sec before vasopressin (Table 4). Ionomycin at concentrations which produced small elevations in $[Ca^{2+}]_i$ (Table 4) and which did not induce platelet aggregation (as monitored turbidometrically) or ATP secretion, slightly enhanced the rate and extent of vasopressin-induced platelet aggregation (Fig. 38). However, at these concentrations, ionomycin failed to convert the vasopressin-induced reversible response ('primary' aggregation) to an irreversible ('secondary') aggregation associated with secretion (Fig. 38A, B). In contrast, under the same conditions, sub-threshold adrenaline markedly potentiated the vasopressin responses resulting in 'secondary' aggregation and ATP secretion (Fig. 38A). Note that under these experimental conditions, the elevations in $[Ca^{2+}]_i$ induced by ionomycin in combination with vasopressin were greater than or equivalent to those produced by vasopressin

TABLE 4. Effects of ionomycin and vasopressin on platelet $[Ca^{2+}]_i$.

Ionomycin (nM)	Increase in $[Ca^{2+}]_i$ (nM)	
	Without VP	With VP
0	-	70 ± 15
10	15 ± 3	80 ± 15
50	50 ± 10	180 ± 45
100	210 ± 45	300 ± 60

Samples (2 ml) were incubated with vasopressin (1 nM) and/or ionomycin at the concentrations indicated and $[Ca^{2+}]_i$ was monitored as described in Methods Section. DMSO (0.5%), the ionomycin vehicle, had no effect on vasopressin-induced elevation of $[Ca^{2+}]_i$. The data are means and standard errors from three experiments using blood from different donors.

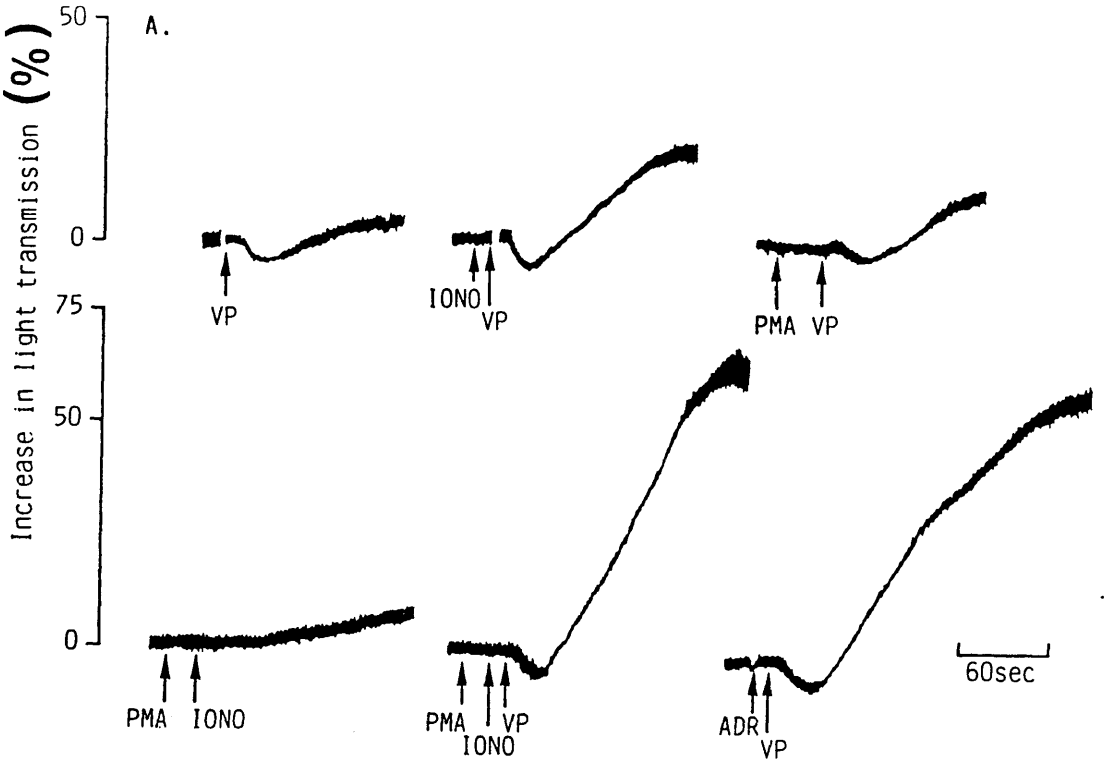

Figure 38(A). Effects of ionomycin and PMA on vasopressin-induced aggregation of Quin 2-loaded gel-filtered platelets - typical aggregometer traces.

Vasopressin, VP (1 nM) was added to stirred samples of Quin 2-loaded platelets which had been incubated with ionomycin (100 nM) and/or PMA (10 nM) for the times indicated and aggregation was monitored photometrically as described in Methods Section. DMSO (0.5%), the vehicle for ionomycin and PMA had no significant effect on vasopressin-induced platelet aggregation. The effect of adrenaline pre-incubation is shown for comparison.

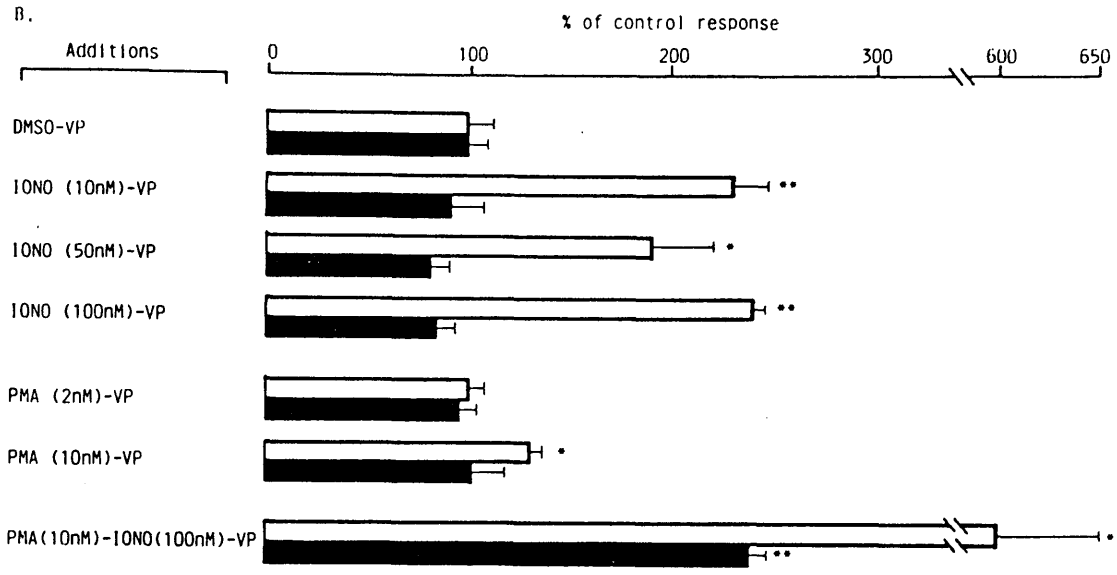

Figure 38(B). Effects of ionomycin and PMA on vasopressin-induced aggregation and ATP secretion of Quin 2-loaded gel-filtered platelets - bar graphs.

Vasopressin was added to stirred samples of Quin 2-loaded gel-filtered platelets which had been incubated with DMSO (0.5%), ionomycin (100 nM) and/or PMA (10 nM) and the initial rate of aggregation (open bars) and the degree of ATP secretion (solid bars) were determined as described in Methods Section. The data are means and standard errors from five different experiments. Data were compared to DMSO controls using Student's t-test: *P <0.05; **P <0.005.

in combination with adrenaline (Tables 3 and 4).

(d) Effects of PMA and ionomycin on vasopressin-induced platelet activation.

To clarify the role of enhanced inositol phospholipid hydrolysis and DG formation in the potentiation of platelet activation by adrenaline, the effects of PMA (effectively a mimic of endogenous DG) on aggregation and ATP secretion induced by a sub-maximal concentration of vasopressin were examined. Pre-incubation of platelets with sub-threshold concentrations of PMA ($\leqslant$ 10 nM; 30 sec) resulted in a small but significant potentiation of vasopressin-induced primary aggregation with no enhancement of ATP secretion (Fig. 38A, B). Higher concentrations of PMA resulted in full aggregation and ATP secretion, when added alone (not shown). Incubation of platelets with PMA (10 nM) and ionomycin (100 nM) had little direct effect on platelet aggregation (as monitored turbidometrically) or ATP secretion (Fig. 38A). However, the subsequent addition of vasopressin resulted in rapid, irreversible aggregation accompanied by secretion of ATP, and the extent of the response was comparable to that elicited by adrenaline and vasopressin in combination (Fig. 38A, B).

III. Effects of adenylate cyclase stimulants (PGI_2 and PGD_2) on platelet responses

In this study I examined the effects of elevation of cyclic AMP levels on different platelet responses and the underlying transduction processes. The aims of this study were (a) to examine the relative susceptibility of whole blood aggregation

induced by different agonists (which utilise different transduction processes) to inhibition by adenylate cyclase stimulants and (b) to examine the mechanism of inhibition of platelet responsiveness by cyclic AMP and the relative susceptibility of different responses and transduction processes to inhibition by cyclic AMP.

III.1. Effects of PGI_2 and PGD_2 on aggregation in whole blood

(a) Effects of varying prostaglandin pre-incubation time.

Incubation (37°C) of blood samples for 90 sec, 3 or 5 min with PGD_2 (10^{-7}M) or PGI_2 (3×10^{-9}M) resulted in inhibition of ADP-induced platelet aggregation in whole blood. The degree of inhibition was independent of the PGD_2 or PGI_2 pre-incubation time and largely could be overcome by increasing concentrations of ADP (Figs. 39 and 40).

(b) Differential effects of PGI_2 and PGD_2 on agonist concentration-response relationships.

This study examined the effects of single concentrations of the adenylate cyclase stimulants PGD_2 (100 nM) and PGI_2 (3 nM) on whole blood platelet aggregation induced by adrenaline, ADP (both adenylate cyclase inhibitors) and vasopressin and PAF (which do not affect adenylate cyclase activity in intact platelets). Pre-incubation (90 sec; 37°C) with PGD_2 (10^{-7}M) had no significant effect on adrenaline-induced aggregation of platelets in whole blood (Fig. 41A). This concentration of PGD_2 produced an approximate 10-fold shift to the right of the ADP concentration-response curve (Fig. 41B) and, apparently, irreversibly inhibited aggregation induced by PAF and vasopressin

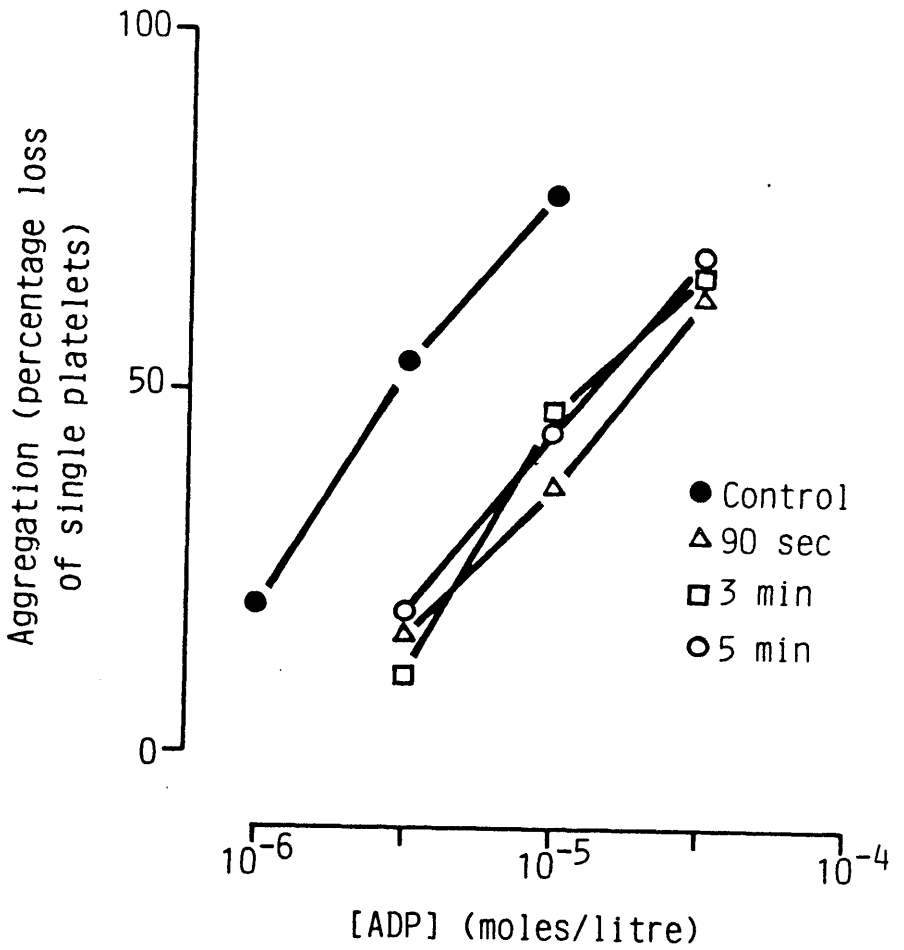

Figure 39. Effects of incubation for 90 seconds, 3 and 5 minutes with PGI_2 on ADP-induced aggregation in whole blood.

ADP-induced platelet aggregation in whole blood was monitored as described in Methods Section in control samples (●) and in samples pre-incubated for 90 seconds (Δ), 3 minutes (□) or 5 minutes (o) with PGI_2 (3 nM). Data are from a single experiment representative of five.

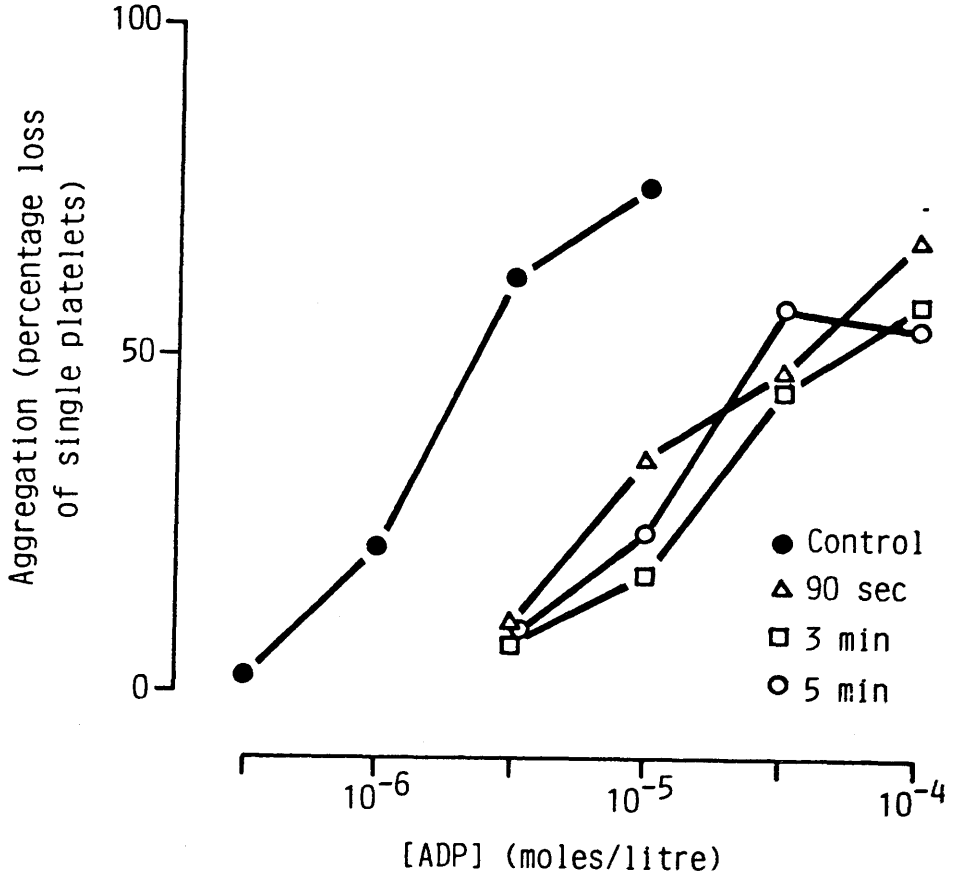

Figure 40. Effects of incubation for 90 seconds, 3 and 5 minutes with PGD_2 on ADP-induced aggregation in whole blood.

ADP-induced platelet aggregation in whole blood was monitored as described in Methods Section in control samples (●) and in samples incubated for 90 seconds (△), 3 minutes (□) or 5 minutes (o) with PGD_2 (100 nM). Data are from a single experiment representative of five.

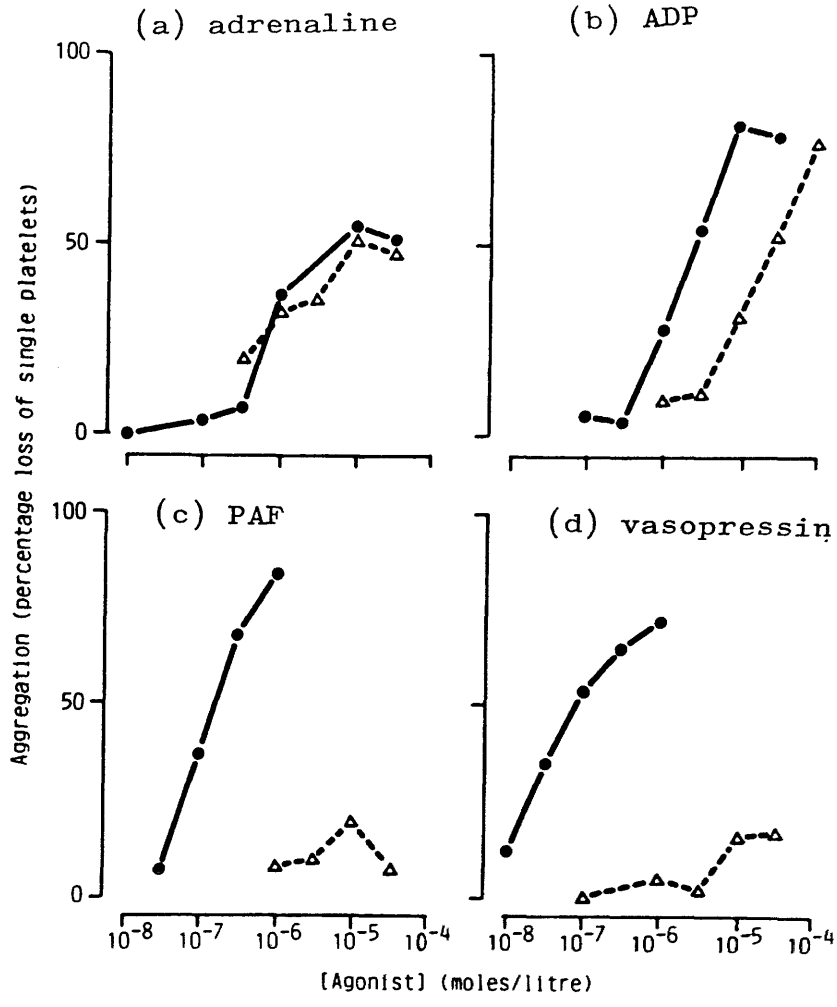

Figure 41. Effects of PGD_2 on agonist-induced platelet aggregation in whole blood.

Aggregation was monitored in response to (a) adrenaline, (b) ADP, (c) PAF and (d) vasopressin in control (●) and PGD_2- (100 nM; 90 second pre-incubation) -treated (Δ) whole blood samples as described in Methods Section. The data are from single experiments representative of at least three others.

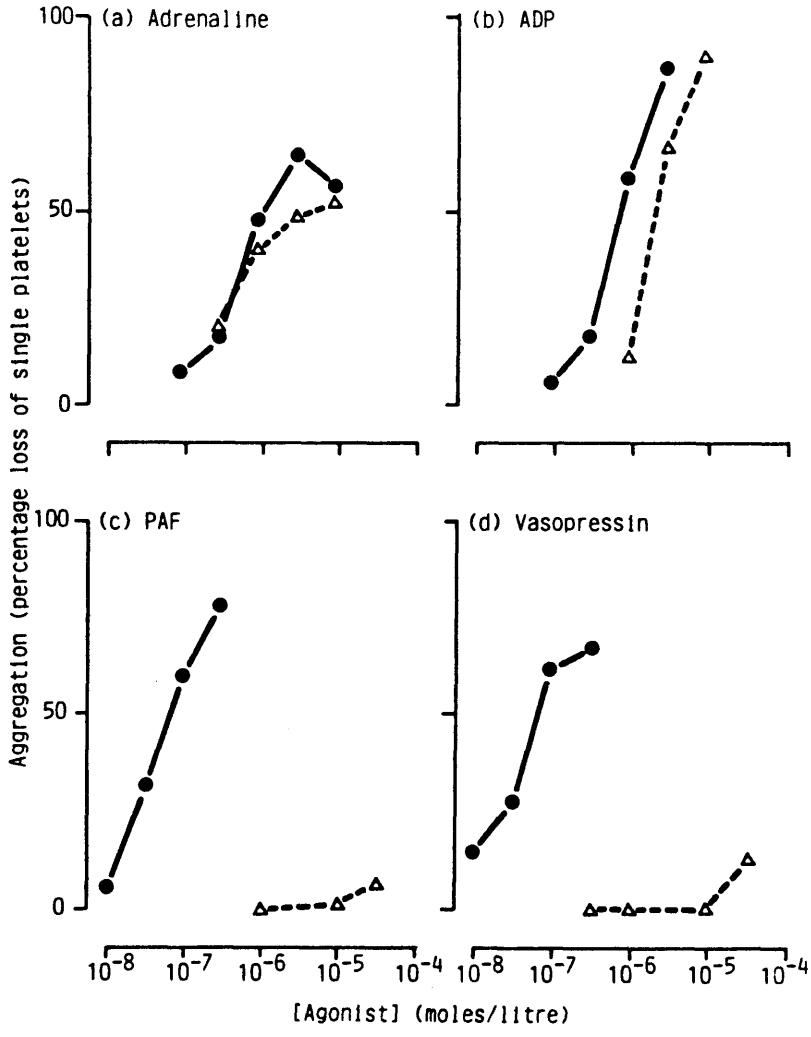

Figure 42. Effects of PGI_2 on agonist-induced platelet aggregation in whole blood.

Aggregation was monitored in response to (a) adrenaline, (b) ADP, (c) PAF and (d) vasopressin in control (●) and PGI_2-(100 nM; 90 second pre-incubation)-treated (Δ) whole blood samples as described in Methods Section. The data are from single experiments representative of at least three others.

(Fig. 41C and D). Similarly, the inhibitory effects of PGI_2 (3×10^{-9}M) on platelet aggregation in whole blood could be overcome by increasing concentrations of adrenaline (Fig. 42a) and ADP (Fig. 42b) but not PAF (Fig. 42c) or vasopressin (Fig. 42d). Higher concentrations of PGI_2 and PGD_2 did inhibit adrenaline-induced aggregation in whole blood (not shown).

III.2. Effects of elevation of platelet cyclic AMP content on agonist-induced platelet responses in Quin 2-loaded, gel-filtered platelets

In order to determine the relationship between elevation of cyclic AMP content and the degree of inhibition of distinct platelet functional responses and transduction processes, the effects of PGD_2 on cylcic AMP content and PAF-induced shape change, aggregation, ATP secretion, stimulation of $[^{32}P]$-Ptd OH formation and elevation of $[Ca^{2+}]_i$ were examined. Incubation with 18 nM PAF resulted in sub-maximal aggregation, ATP secretion, shape change, $[^{32}P]$-Ptd OH formation and elevations in $[Ca^{2+}]_i$ (as described in Sections I.3 - I.5). PGD_2 (1 - 300 nM) caused concentration-dependent inhibition of PAF (18 nM)-induced aggregation, ATP-secretion, shape change, $[^{32}P]$-Ptd OH formation and elevation of $[Ca^{2+}]_i$ monitored both in the presence and in the absence of EGTA (Fig. 43). PGD_2 was more potent as an inhibitor of PAF-induced ATP secretion (I_{50}, i.e. the concentration causing 50% inhibition, $\sim$ 2 nM) and aggregation ($I_{50} \sim$ 3 nM) than of $[^{32}P]$-Ptd OH formation ($I_{50} \sim$ 10 nM), shape change ($I_{50} \sim$ 30 nM) and elevation of $[Ca^{2+}]_i$ ($I_{50} \sim$ 30 nM). Mobilisation of internal Ca^{2+} (i.e. monitored in the presence

of EGTA, 4 mM) and the elevation of $[Ca^{2+}]_i$ that results from both influx and mobilisation (i.e. monitored in the presence of $[Ca^{2+}]_o$ = 1 mM) were equally susceptible to inhibition by PGD_2 (Fig. 43e and f). During these experiments, sub-samples (0.5 ml) of platelets were removed from the aggregometer or fluorimeter cuvettes prior to the addition of PAF, for the estimation of cyclic AMP content. In the phospholipid studies, cyclic AMP content was monitored in parallel samples which had not been treated with $[^{32}P]$-P_i. The effects of PGD_2 on cyclic AMP content under these conditions are shown in Figure 44. The degree of inhibition of the various platelet responses could be correlated with the rise in cyclic AMP content elicited by PGD_2 in the same (or parallel samples for $[^{32}P]$-Ptd OH formation) platelet samples (Fig. 45). PAF-induced ATP secretion and aggregation were very susceptible to small increments in the cyclic AMP content, both being abolished by a doubling in the cyclic AMP content. However, $[^{32}P]$-Ptd OH formation, shape change and elevation of $[Ca^{2+}]_i$ were less susceptible: maximal inhibition of these responses did not occur until the cyclic AMP content had been elevated by greater than ten-fold (Fig. 45).

To test whether this apparent increased sensitivity of aggregation over shape change to inhibition by PGD_2 also occurred with other stimulants of platelet adenylate cyclase, gel-filtered platelets were incubated (90 sec; 37°C) with PGI_2 (0.1 - 3 nM) which also resulted in concentration-dependent elevation of platelet cyclic AMP content (Fig. 46). PAF-induced platelet responses were also differentially inhibited by PGI_2: PAF (1.8 nM)-induced shape change was less susceptible to inhibition by PGI_2 than was aggregation induced by 18 nM

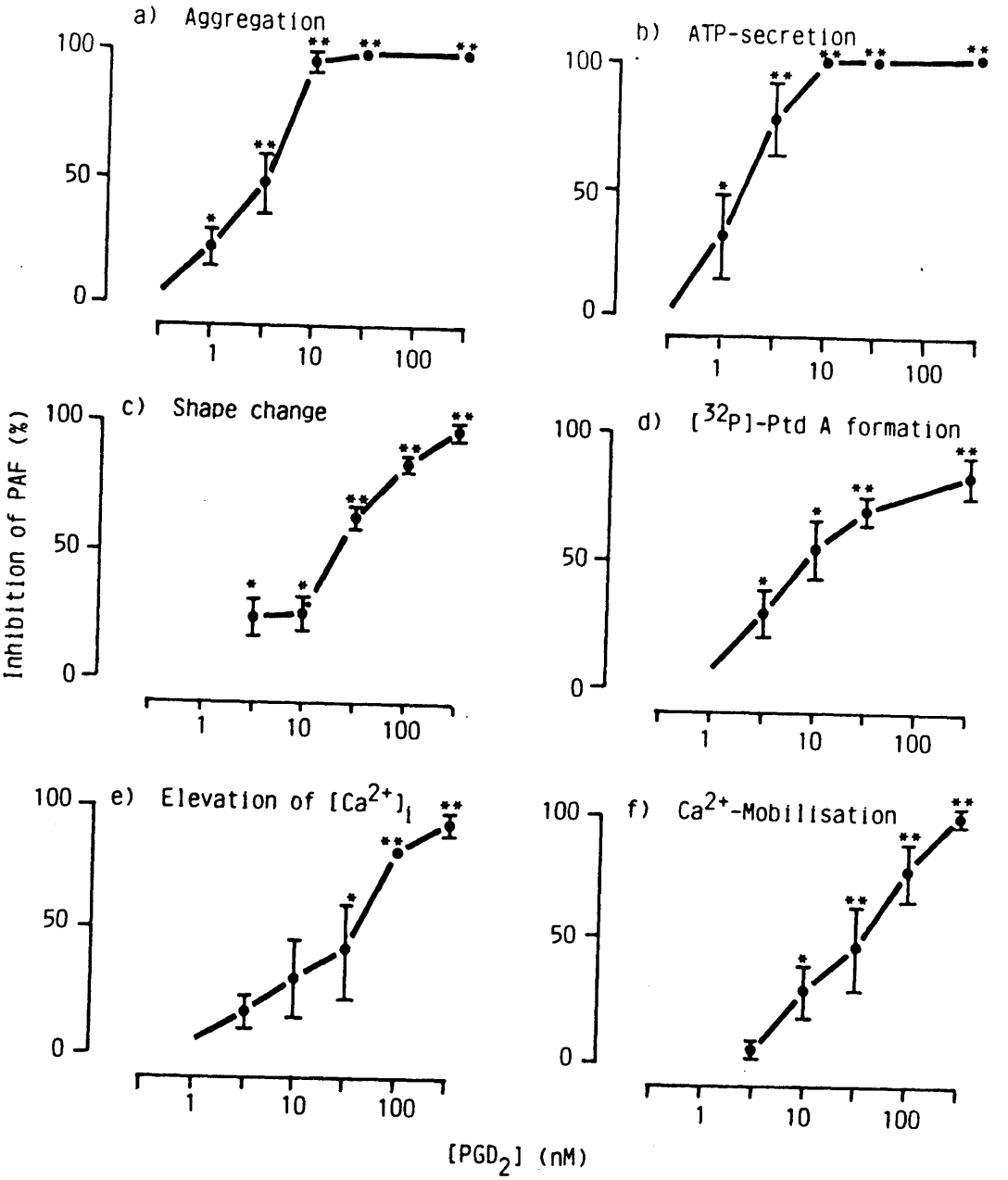

Figure 43. Effects of PGD_2 on PAF-induced platelet responses.

Plasma-free platelet suspensions, prepared as described in Methods Section, were incubated with PGD_2 (90 sec; 37°C) at the concentrations indicated before the addition of PAF (18-180 nM). Inhibition of PAF-induced (a) aggregation, (b) ATP-secretion, (c) shape change, (d) $[^{32}P]$-Ptd OH formation, (e) elevation of $[Ca^{2+}]_i$ in the presence of 1 mM- $[Ca^{2+}]_o$ and (f) Ca^{2+} mobilization in the presence of EGTA (4 mM) were monitored as described in Methods Section. Note that $[Ca^{2+}]_i$ changes in part (e) result from mobilisation of internal Ca^{2+} stores and influx of external Ca^{2+} whilst those in part (f) result from mobilisation of internal Ca^{2+} stores alone. The data are mean values and standard errors from between two and seven different experiments each performed in triplicate. The data were compared to control values using Student's t-test: $*P < 0.05$; $**P < 0.005$.

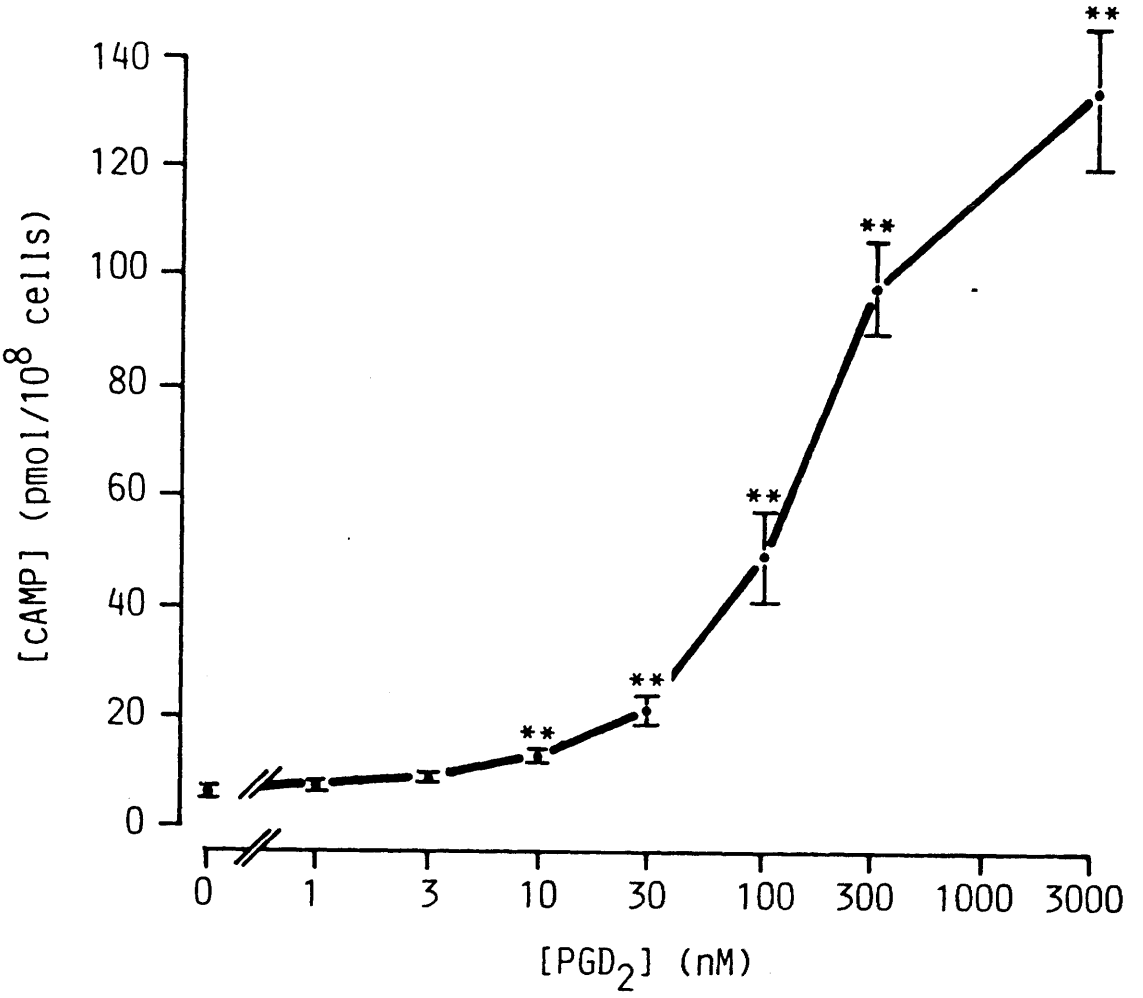

Figure 44. Effects of PGD_2 on platelet cyclic AMP content.

Plasma-free platelet samples (0.2 - 0.4 ml) were incubated(90 seconds; 37°C) with PGD_2 at the concentrations indicated and cyclic AMP content was measured as described in Methods Section. The data are mean values and standard errors from sixteen different experiments each performed in triplicate and results were compared to control values using Student's t-test: *$p < 0.05$; **$p < 0.005$.

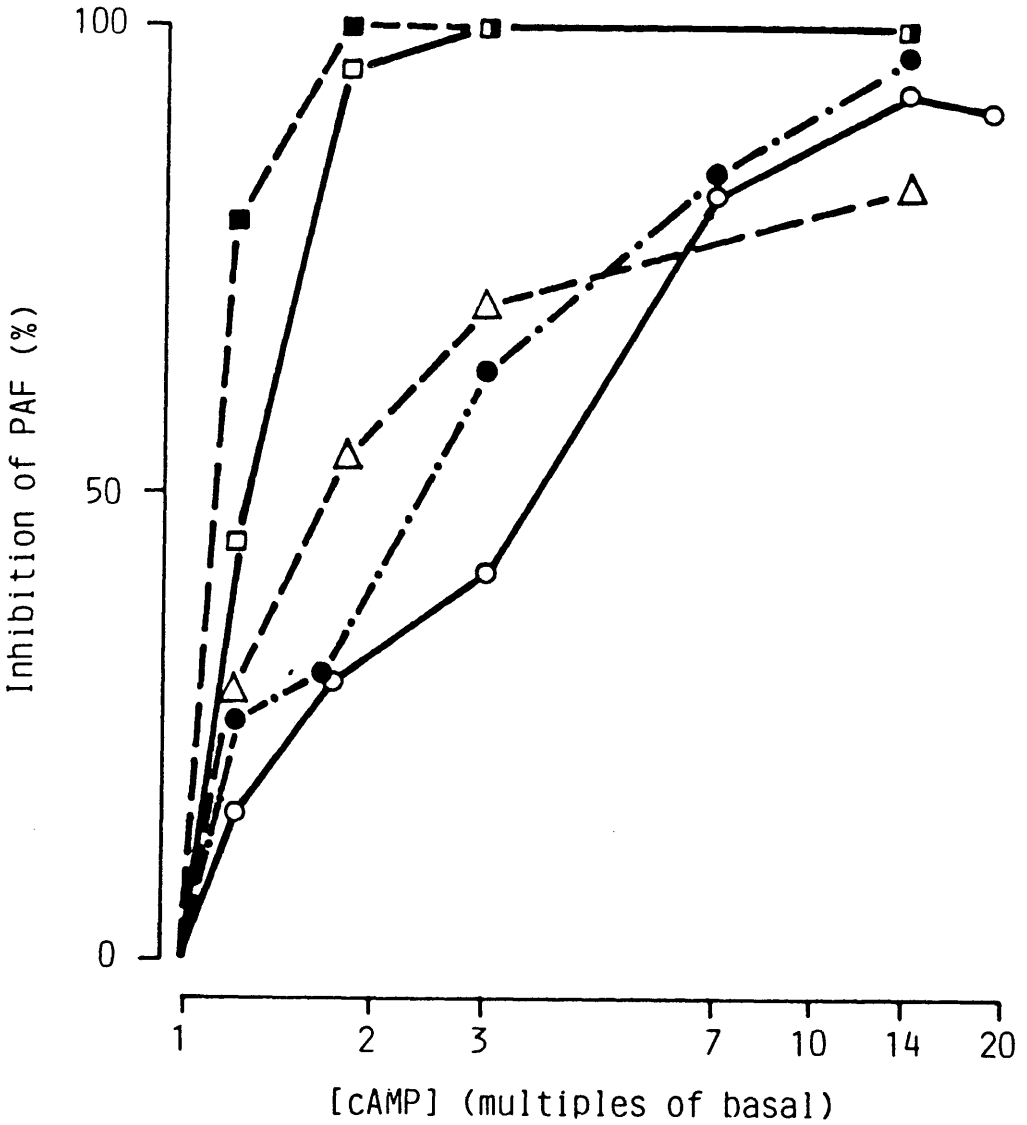

Figure 45. Relationship between platelet cyclic AMP content and inhibition of PAF-induced platelet responses.

Plasma-free platelet suspensions were incubated with PGD_2 (1-300 nM; 90 seconds; 37°C) before the addition of PAF (18 - 180 nM) and/or removal of a sample for estimation of cyclic AMP content. Inhibition of PAF-induced ATP-secretion (■), aggregation (□), $[^{32}P]$-PtdOH formation (Δ), shape change (●) and elevation of $[Ca^{2+}]_i$ (o) are shown as a function of the fold-rise in cyclic AMP content of each platelet sample (or parallel samples for the phospholipid studies where $[^{32}P]$-P_i was omitted). The basal cyclic AMP content was 6.9 ± 0.4 pmoles per 10^8 platelets. The data are mean values from 2-7 experiments and error bars were omitted for the sake of clarity.

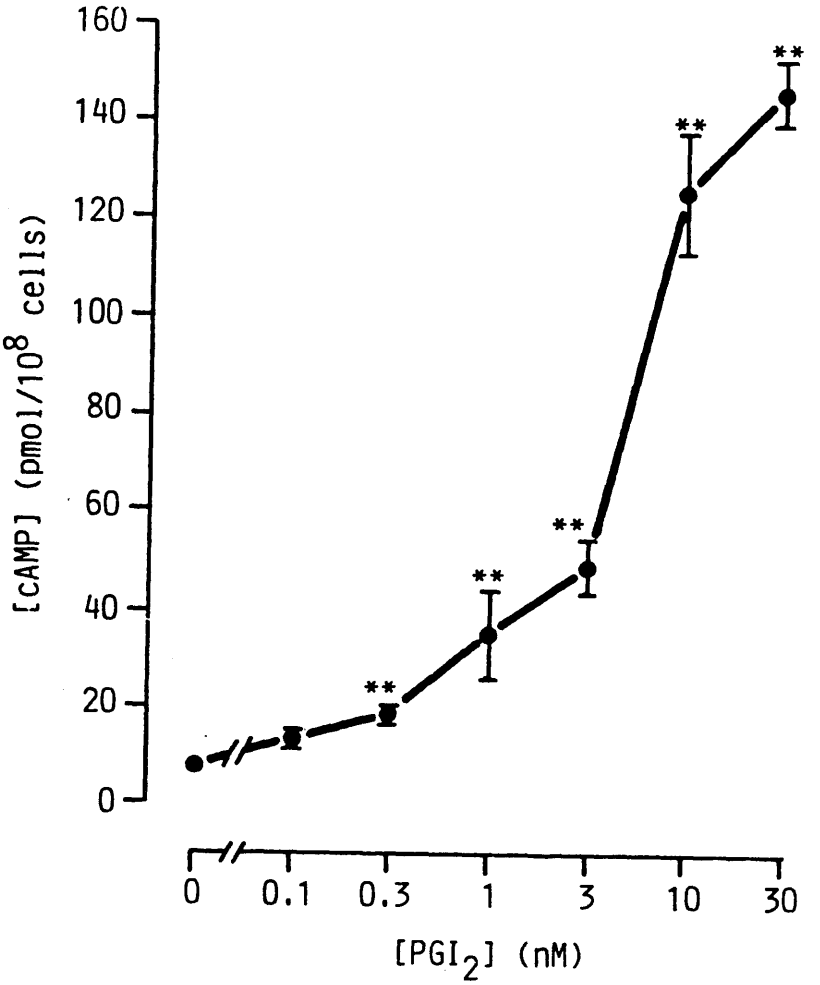

Figure 46. Effects of PGI_2 on platelet cyclic AMP content.

Gel-filtered platelets (0.4 ml) were incubated with PGI_2 (90 seconds; 37°C) at the concentrations indicated and cyclic AMP content was determined as described in Methods Section. The data are mean values and standard errors from three different experiments and results were compared to control values using Student's t-test: *$p < 0.005$.

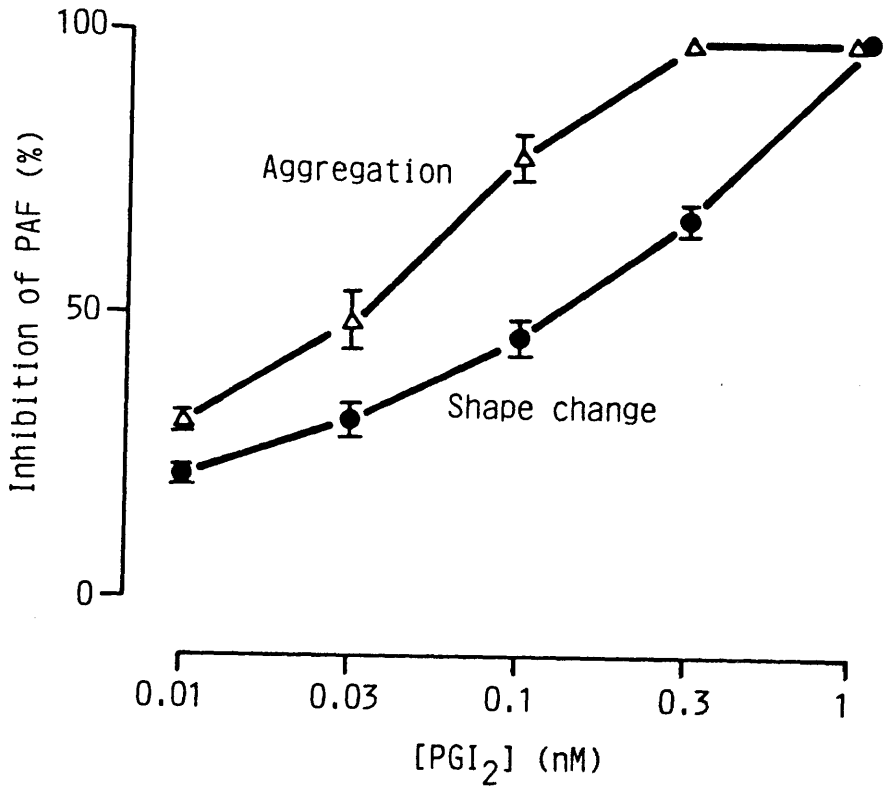

Figure 47. Effects of PGI_2 on PAF-induced platelet aggregation and shape change.

Gel-filtered platelets were incubated with PGI_2 (90 sec; 37°C) at the concentrations indicated and PAF (18 nM)-induced aggregation (Δ) and shape change (●) were monitored as described in Methods Section. The data are from a single experiment representative of three and show inhibition of the extent of PAF (18 nM)-induced aggregation and ATP secretion.

PAF in parallel samples (Fig. 47).

IV. Reversal of prostaglandin-induced elevations in cyclic AMP content and inhibition of platelet function by stimulatory agonists

The effects of combinations of platelet stimulatory and inhibitory agonist on cyclic AMP levels and various platelet responses were investigated. The aim of this study was to examine the regulation of platelet responsiveness and transduction processes by cyclic AMP in the presence of multiple agonists.

IV.1. Effects of adrenaline and vasopressin on inhibition of PAF-induced platelet aggregation in whole blood by PGI_2 and PGD_2

Inhibition by PGD_2 of PAF-induced platelet aggregation in whole blood was reversed by adrenaline (10^{-7}M) added 30 sec after the PGD_2 (Fig. 48). This concentration of adrenaline produced 5 $\pm$ 3% aggregation when added, alone, to blood samples. In contrast, the inhibition of PAF-induced aggregation by PGD_2 was not reversed by vasopressin (3×10^{-7}M) (Fig. 48). This concentration of vasopressin, when added alone, produced 60 $\pm$ 5% aggregation. Similarly, inhibition of PAF-induced aggregation by PGI_2 (3×10^{-9}M) could be reversed by adrenaline (10^{-7}M) but not by vasopressin (10^{-6}M) (Fig. 49).

IV.2. Effects of agonists on PGI_2-induced inhibition of platelet function and elevations in cyclic AMP content in Quin 2-loaded, gel-filtered platelets

Aggregation, ATP secretion and elevation of $[Ca^{2+}]_i$ in response to PAF (18 nM) were significantly inhibited in platelets

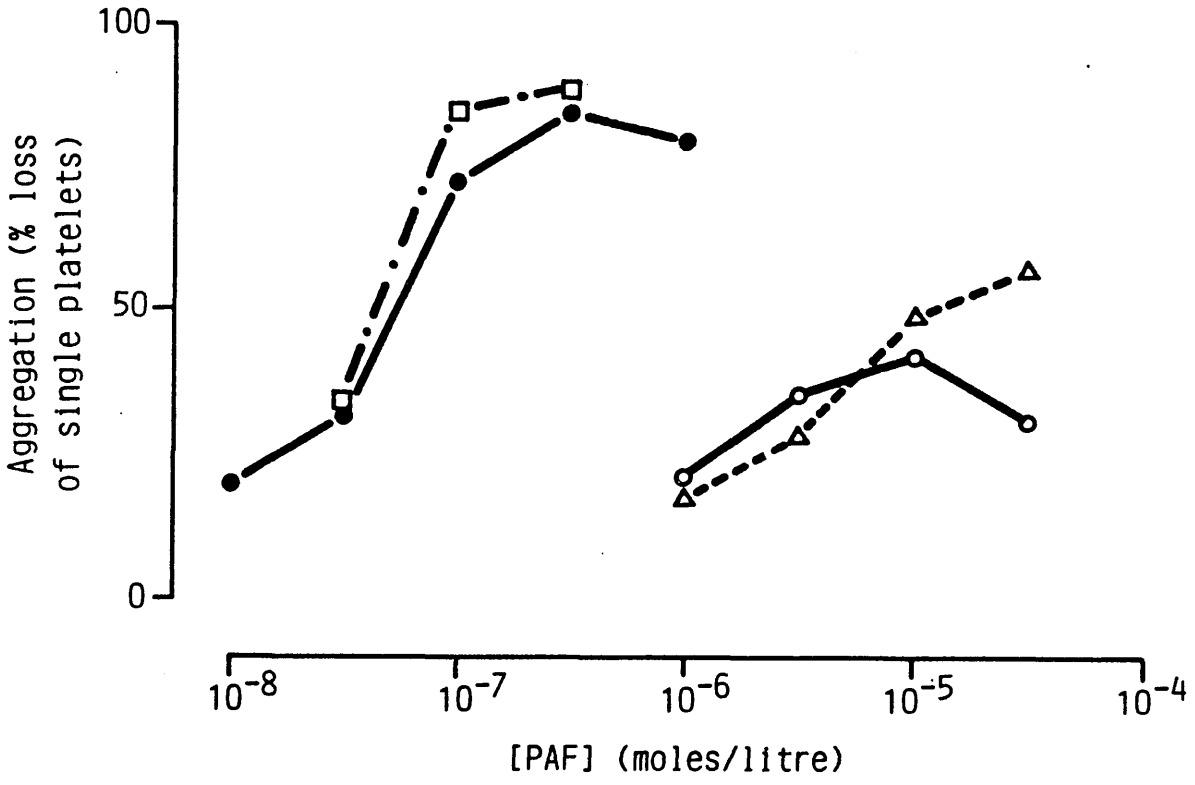

Figure 48. Effects of vasopressin and adrenaline on the inhibition of PAF-induced aggregation in whole blood by PGD_2.

PAF-induced whole blood platelet aggregation was monitored as described in Methods Section in control samples (●); samples incubated with PGD_2: 100 nM, 90 seconds (△); PGD_2: 100 nM, 90 seconds plus vasopressin: 300 nM, 60 seconds (o) or PGD_2: 100 nM, 90 seconds plus adrenaline: 100 nM, 60 seconds (□). The data are from a single experiment representative of four.

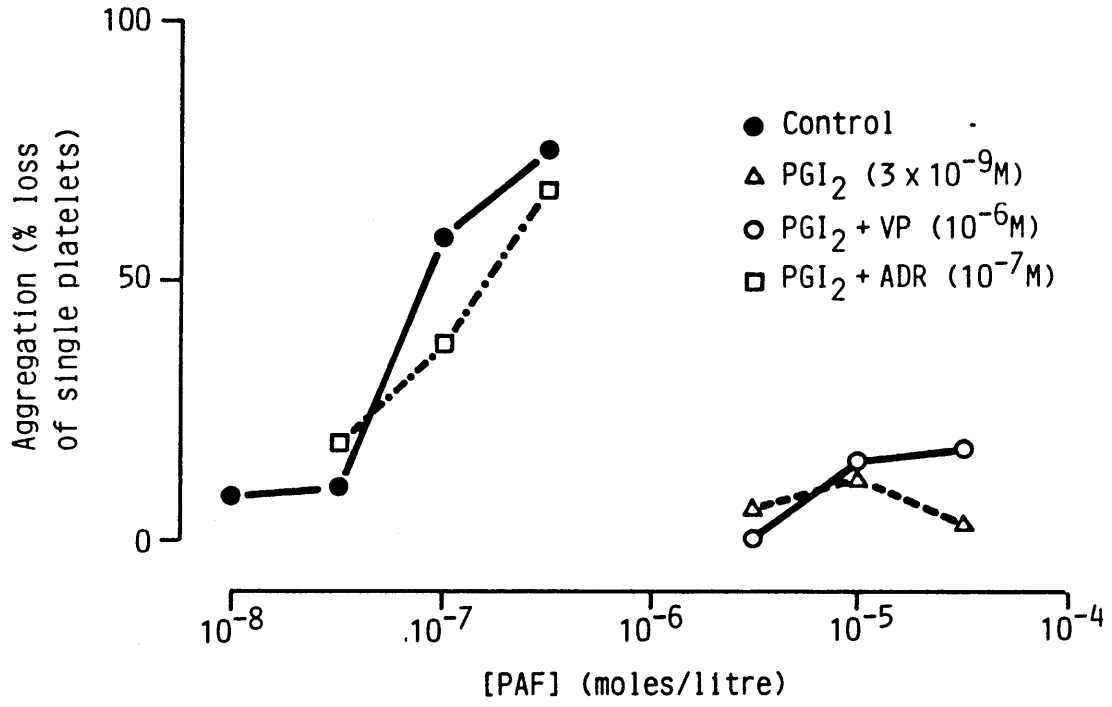

Figure 49. Effects of vasopressin and adrenaline on the inhibition of PAF-induced aggregation in whole blood by PGI_2.

PAF-induced whole blood platelet aggregation was monitored as described in Methods Section in control samples (●); samples incubated with PGI_2: 3 nM, 90 seconds (△); PGI_2: 3 nM, 90 seconds plus vasopressin: 1 μM, 60 seconds (○) or PGI_2: 3 nM, 90 seconds plus adrenaline: 100 nM, 60 seconds (□). The data are from a single experiment representative of four.

pre-incubated (90 sec; 37°C) with PGI_2 (5 nM) (Fig. 50). The inhibitory effects of PGI_2 on these responses were reversed by adrenaline (5μM) and ADP (5 μM) but not by vasopressin (2 μM) (Fig. 50). Vasopressin (30 nM)-induced aggregation, ATP-secretion, elevation of $[Ca^{2+}]_i$ and $[^{32}P]$-PtdOH formation were also inhibited in platelets treated (90 sec; 37°C) with PGI_2 (5 nM) (Fig. 51). Inhibition of vasopressin-induced platelet responses was also prevented by adrenaline (5 μM) (Fig. 51). Under these conditions, inhibition of platelet function by PGI_2 was associated with elevation of the platelet cyclic AMP content from the basal value of around 5 pmoles per 10^8 platelets to 50-60 pmoles per 10^8 platelets (Fig. 52). This PGI_2-induced elevation in cyclic AMP content was prevented by adrenaline (5 μM) and ADP (5 μM) but was unaffected by vasopressin (2 μM) or PAF (900 nM) (Fig. 52).

IV.3. Effects of adrenaline on inhibition of platelet function by IBMX and 8 bromo-cyclic AMP

Incubation of gel-filtered platelets (30 min; 37°C) with the cyclic AMP phosphodiesterase inhibitor IBMX (1 mM) resulted in an approximate doubling of the platelet cyclic AMP content. The IBMX-induced rise in cyclic AMP content was not significantly altered by further incubation (90 sec; 37°C) of platelets with adrenaline (5 μM) (Table 5). Under these conditions, IBMX inhibited aggregation and ATP secretion induced by PAF (18 nM) and these effects were not attenuated by adrenaline (5 μM) (Fig. 53). Similar findings were obtained using 8 bromo-cyclic AMP (data not shown). In addition, PAF (18 nM)-induced elevation of $[Ca^{2+}]_i$ was suppressed by pre-incubation (30 min;

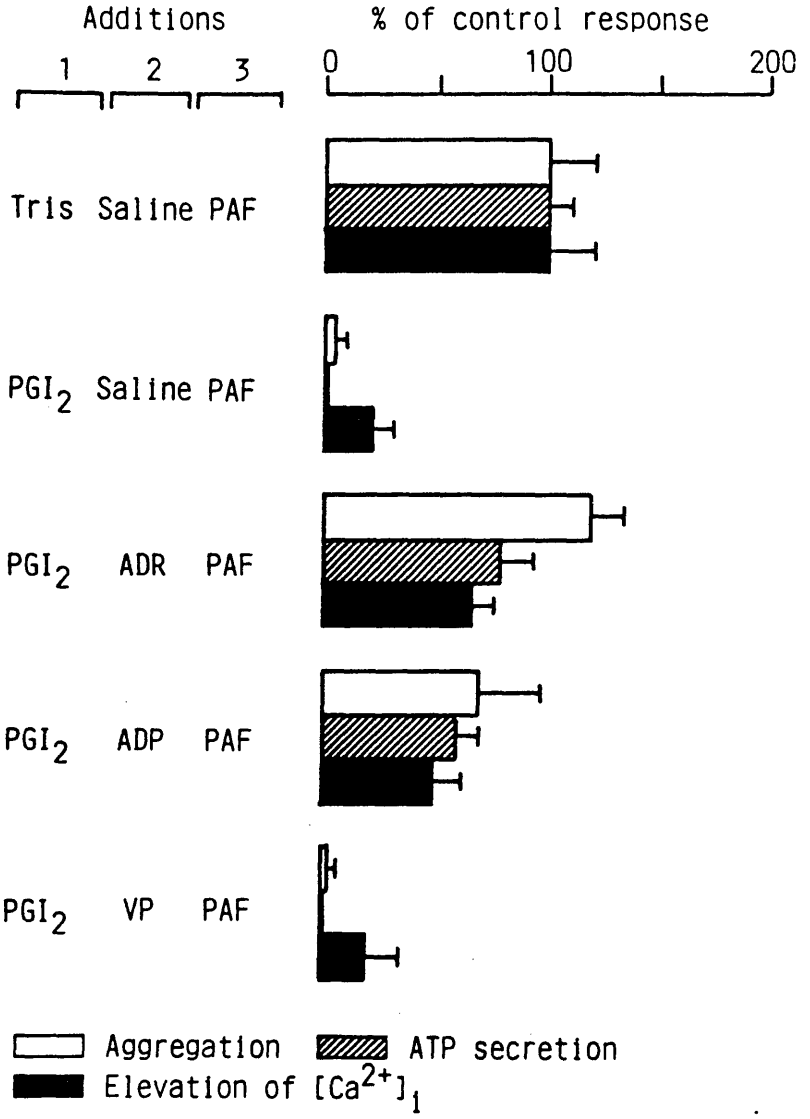

Figure 50. Effects of adrenaline, ADP and vasopressin on the inhibition of PAF-induced aggregation, ATP secretion and elevation of $[Ca^{2+}]_i$ by PGI_2.

PAF (18 nM)-induced aggregation (open bars), ATP secretion (hatched bars) and elevation of $[Ca^{2+}]_i$ (solid bars) were monitored in parallel in Quin 2-loaded gel-filtered platelets as described in Methods Section. Where indicated, platelets were pre-treated with PGI_2 (3 nM) or vehicle (Tris pH 9) for 90 seconds and saline, adrenaline (5 μM), ADP (5 μM) or vasopressin (2 μM) for 60 seconds. The data are means and standard errors from a single experiment (representative of three) performed in triplicate.

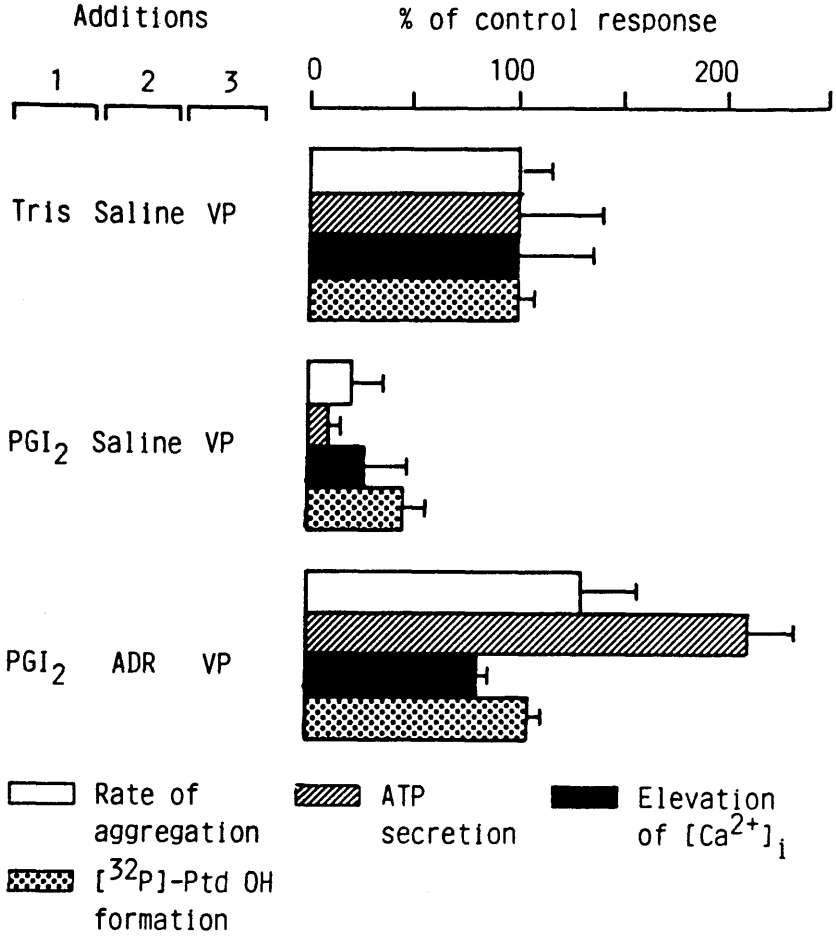

Figure 51. Effects of adrenaline on the inhibition of vasopressin-induced aggregation, ATP secretion, elevation of $[Ca^{2+}]_i$ and $[^{32}P]$-Ptd OH formation.

Vasopressin (30 nM)-induced aggregation (open bars), ATP-secretion (hatched bars), elevation of $[Ca^{2+}]_i$ (solid bars) and $[^{32}P]$-Ptd OH formation (dotted bars) were monitored in plasma-free platelet suspensions as described in Methods Section. Where indicated, platelets were pre-treated with PGI_2 (5 nM) or vehicle (Tris pH 9) for 90 seconds and saline or adrenaline (5 μM) for 60 seconds. The data are means and standard errors from single experiments (representative of at least two similar experiments) performed in triplicate.

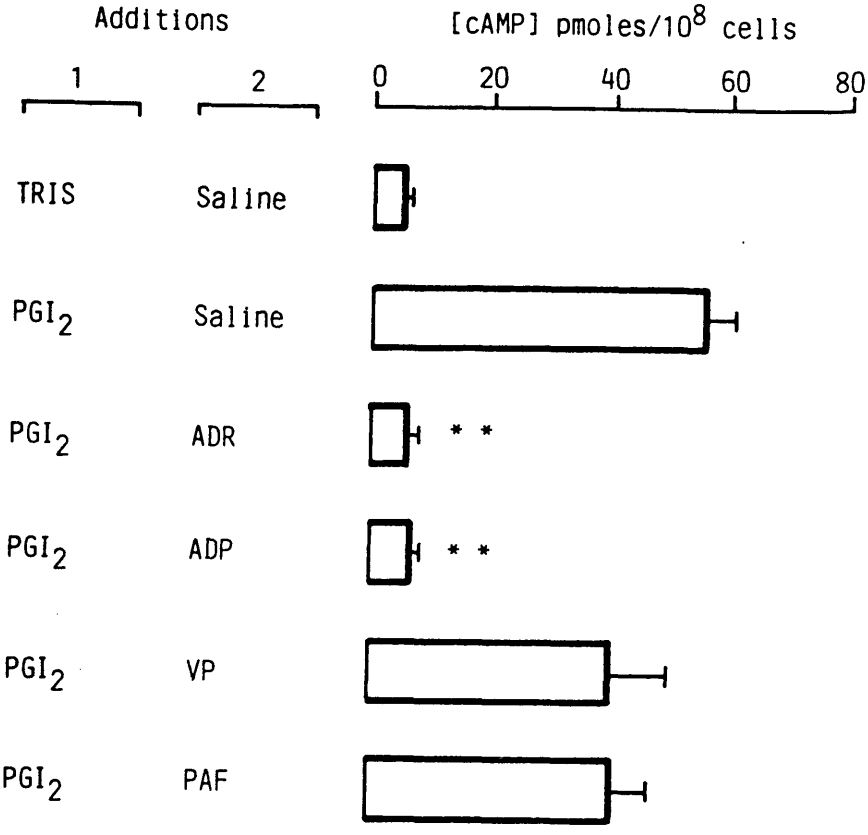

Figure 52. Effects of agonists on PGI_2-induced elevation of platelet cyclic AMP content.

Gel-filtered platelets (0.4 ml) were incubated (37°C) with PGI_2 (5 nM) or vehicle (Tris pH 9) for 90 seconds and with saline, adrenaline (5 μM), ADP (5 μM), vasopressin (2 μM) or PAF (900 nM) for 60 seconds. Cyclic AMP content was monitored as described in Methods Section. The data are means and standard errors from a single experiment (representative of three) performed in triplicate. Agonist-treated samples were compared to the PGI_2-saline control using Student's t-test: $^{**}P < 0.005$. Adrenaline, ADP, vasopressin or PAF had no significant effect on the basal cyclic AMP content when added alone (not shown).

TABLE 5. Effects of adrenaline and IBMX on platelet cyclic AMP content.

Treatment	Cyclic AMP content (pmol/10^8 platelets)
Control	8.6 ± 0.4
IBMX (1 mM)	19.8 ± 3.2
IBMX + Adrenaline (1mM) (5 μM)	17.8 ± 3.7
Adrenaline (5 μM)	8.4 ± 0.3

Control or IBMX (1 mM; 10-30 min; 37°C)-treated gel-filtered platelet samples (0.4 ml) were incubated (30 sec; 37°C) with saline or adrenaline (5 μM) and cyclic AMP content was measured as described in Methods Section. The data are means and standard errors from a single experiment representative of three similar.

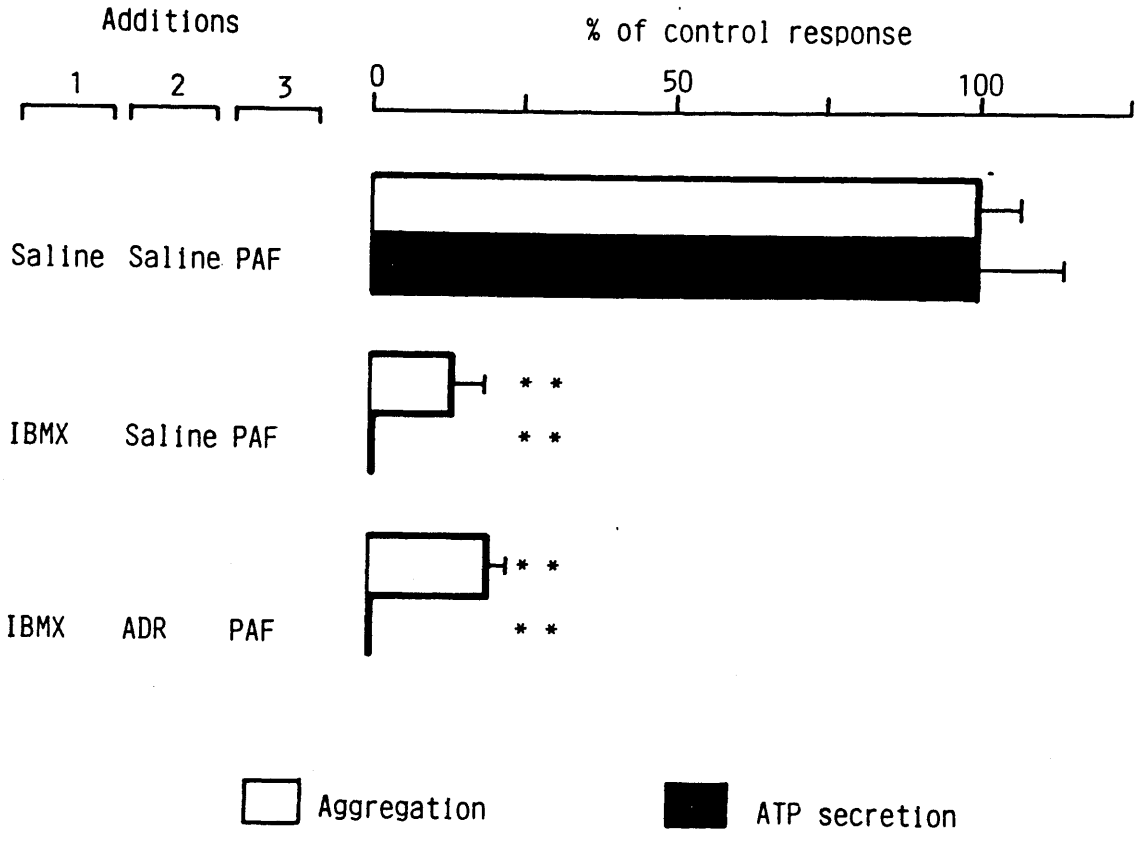

Figure 53. Effects of adrenaline on the inhibition of PAF-induced aggregation and ATP secretion by IBMX.

PAF (18 nM)-induced aggregation (open bars) and ATP secretion (hatched bars) were monitored in gel-filtered platelets as described in Methods Section. Where indicated, platelets were pre-incubated with IBMX (1 mM; 10-30 min; 37°C) and saline or adrenaline (5 μM; 30 sec; 37°C). The data are means and standard errors from a single experiment (representative of three) performed in triplicate. Results were compared to the PAF control using Student's t-test: $^{**}p < 0.005$.

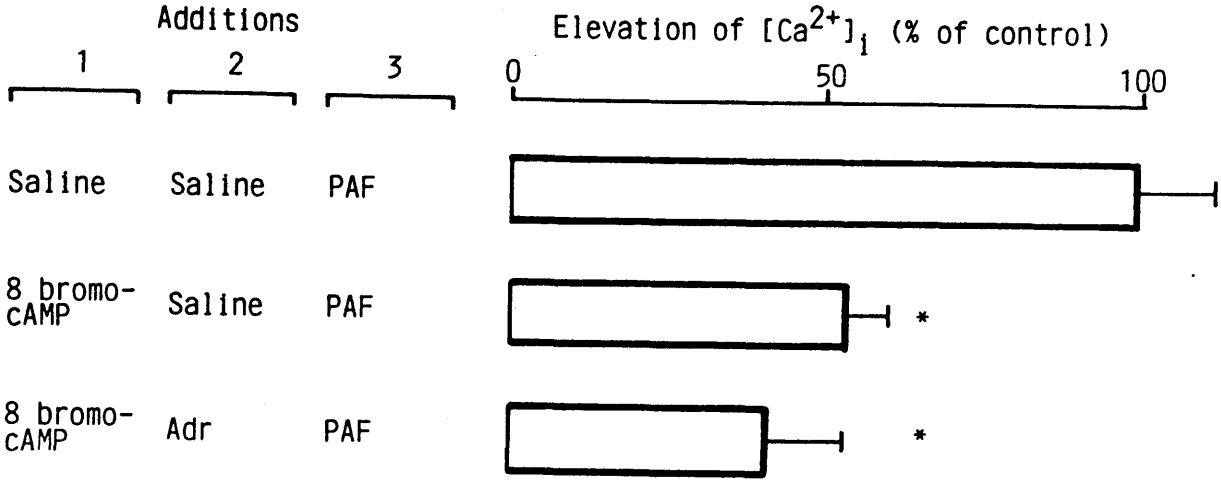

Figure 54. Effects of adrenaline on the inhibition of PAF-induced elevation of $[Ca^{2+}]_i$ by 8 bromo-cyclic AMP.

PAF (18 nM)-induced elevation of $[Ca^{2+}]_i$ was monitored in Quin 2-loaded gel-filtered platelets as described in Methods Section. Where indicated, the platelets were pre-incubated (37°C) with 8-bromo-cyclic AMP (1 mM; 30 min) and saline or adrenaline (5 μM; 30 sec). The data are means and standard errors from a single experiment (representative of three) performed in triplicate. Results were compared to PAF control using Student's t-test: *$P < 0.05$.

37°C) with 8 bromo-cyclic AMP (1 mM) and this inhibitory effect was not modified by adrenaline (5 μM) (Fig. 54).

V. Effects of activation of protein kinase C by PMA on platelet cyclic AMP metabolism

This study examined the possible regulation of platelet cyclic AMP metabolism by activation of protein kinase C.

V.1. Effects of phorbol esters on basal, IBMX- and PGD_2-elevated platelet cyclic AMP content

Incubation (4 min; 37°C) of gel-filtered platelets with PMA (3 - 300 nM) had no significant effect on the cyclic AMP content of control (untreated) or IBMX (1mM)-treated platelets (Fig. 55). However, elevation of platelet cyclic AMP content induced by PGD_2 (300 nM; 2 min) was inhibited, in a concentration-dependent manner, by pre-incubation (2 min; 37°C) with PMA (3 - 300 nM). The I_{50} for this effect was around 7.5 nM. This effect of PMA was not shared by the inactive, non-tumour-promoting, phorbol ester 4αPDD (Fig. 56).

As PMA is known to induce platelet activation, degranulation and eicosanoid biosynthesis, it was possible that the effects of the tumour-promoting phorbol ester on agonist-induced cyclic AMP formation were mediated by agents (e.g. ADP, TxA_2) released by platelets and which are claimed to inhibit adenylate cyclase. To investigate the role of PMA-induced TxA_2 formation in this effect, platelets were incubated (15 - 60 min; 37°C) with the cyclo-oxygenase inhibitor flurbiprofen (10 μM) to prevent the biosynthesis of prostanoids. Flurbiprofen had no significant effect on the inhibition of the PGD_2-induced rise in cyclic AMP

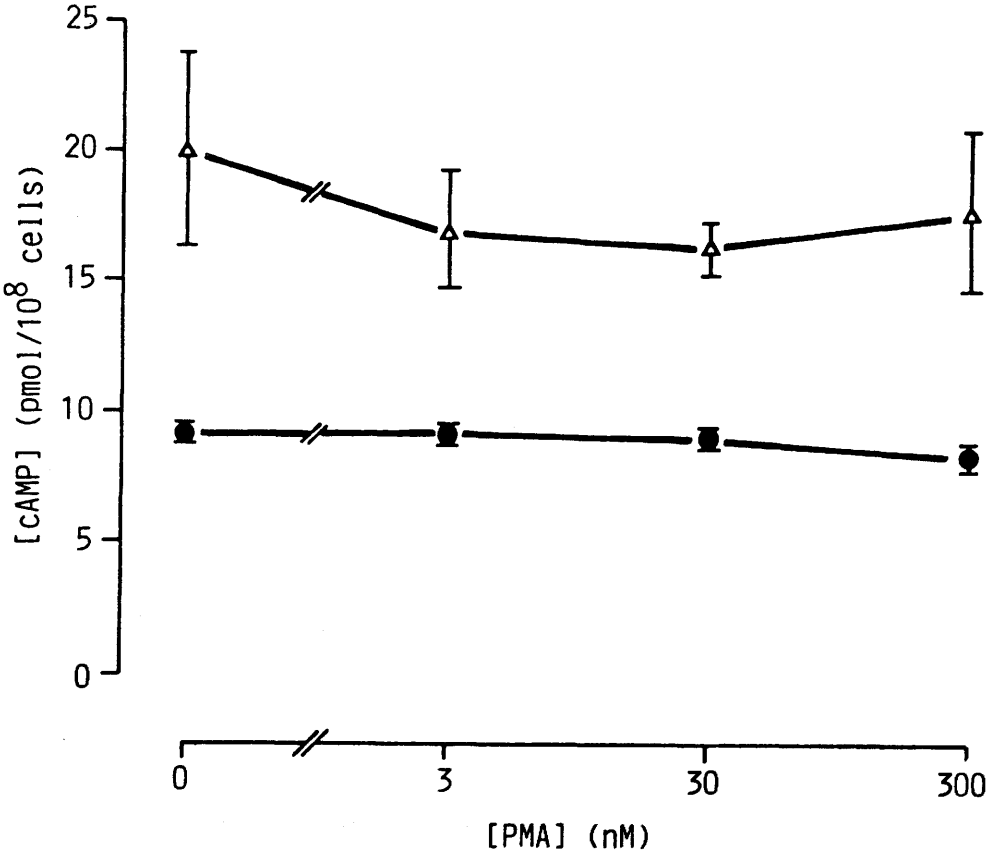

Figure 55. Effects of PMA on basal and IBMX-elevated cyclic AMP content.

Control (●) and IBMX (1 mM; 30-60 min)-treated gel-filtered platelet samples (△) were incubated (4 min; 37°C) with PMA at the concentrations indicated and cyclic AMP content was monitored as described in Methods Section. The data are means and standard errors from three different experiments.

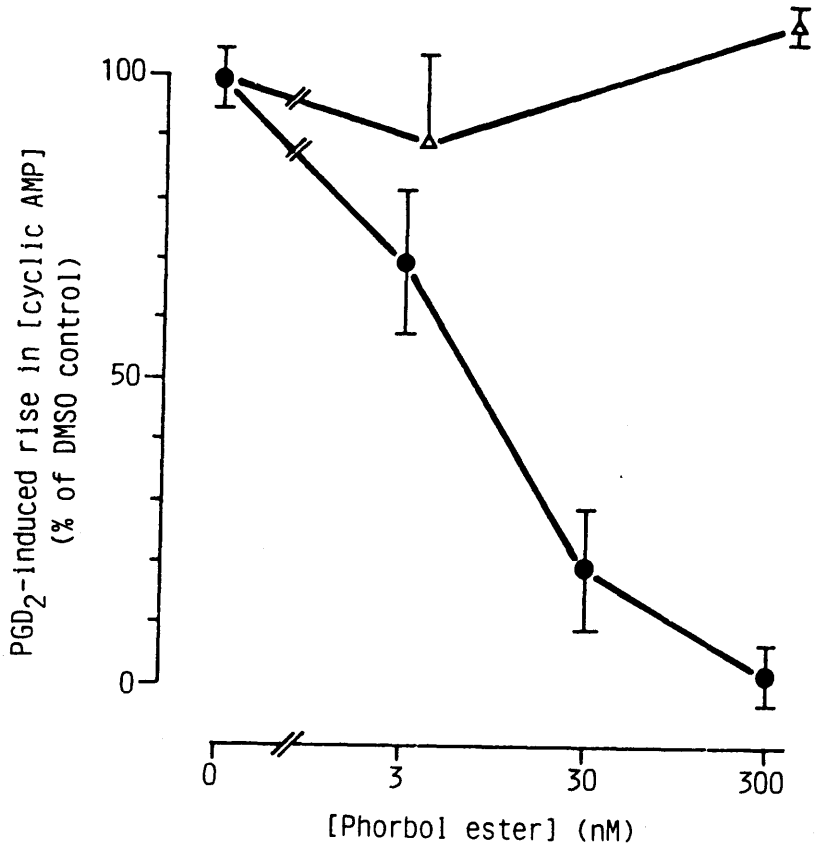

Figure 56. Effects of PMA and 4 αPDD on PGD_2-induced elevation of platelet cyclic AMP content.

Gel-filtered platelet samples (0.4 ml) were incubated (37°C) with PMA (●) or 4 αPDD (Δ) at the concentrations indicated for 2 minutes before the addition of PGD_2 (300 nM) for a further 2 minutes. Cyclic AMP content was monitored as described in Methods Section. For the sake of comparison between different experiments, results are expressed as percentage of the control rise in cyclic AMP content induced by PGD_2 (300 nM) in the presence of the PMA vehicle, DMSO (0.25%). The basal cyclic AMP content was 8.3 ± 0.4 nM and this was raised to 124 ± 10 nM in the presence of PGD_2 (300 nM). The data are means and standard errors from three different experiments.

although there was a slight increase in the I_{50} to around 10 nM (Fig. 57).

The role of PMA-induced ADP release in blocking PGD_2-induced cyclic AMP formation were investigated by treating platelets (10 - 50 min; 37°C) with the ADP scavengers: apyrase (10 units of ADPase activity per ml of gel-filtered platelets) or creatine phosphate (2 mM) plus creatine phosphokinase (50 units per ml). Apyrase (10 units per ml) prevented the inhibition of PGD_2-induced elevation of cyclic AMP by exogenously added ADP (1 - 10 μM) but had no significant effect on the inhibition by PMA (3 - 300 nM). The I_{50} for inhibition by PMA in the presence of apyrase was around 11 nM (Fig. 58). In the presence of creatine phosphate/creatine phosphokinase, PMA inhibited the rise in cyclic AMP content with an I_{50} of around 2.6 nM (Fig. 59).

Thus, PMA-induced inhibition of the rise in platelet cyclic AMP content cannot be attributed to formation or release of endogenous inhibitors of adenylate cyclase. Activation of phosphodiesterase by protein kinase C could contribute, at least in part, to the actions of PMA. Accordingly, the effects of PMA on PGD_2-induced cyclic AMP formation were examined in the presence of IBMX. PGD_2-induced elevation of cyclic AMP content was doubled in platelets incubated (15 - 60 min; 37°C) with IBMX, 1 mM (Table 6). Under these conditions, IBMX only partially inhibited the abrogation of PGD_2-induced elevation of cyclic AMP content by PMA. In the presence of IBMX (1 mM), PMA inhibited PGD_2-induced cyclic AMP formation with an I_{50} of around 30 nM (Fig. 60).

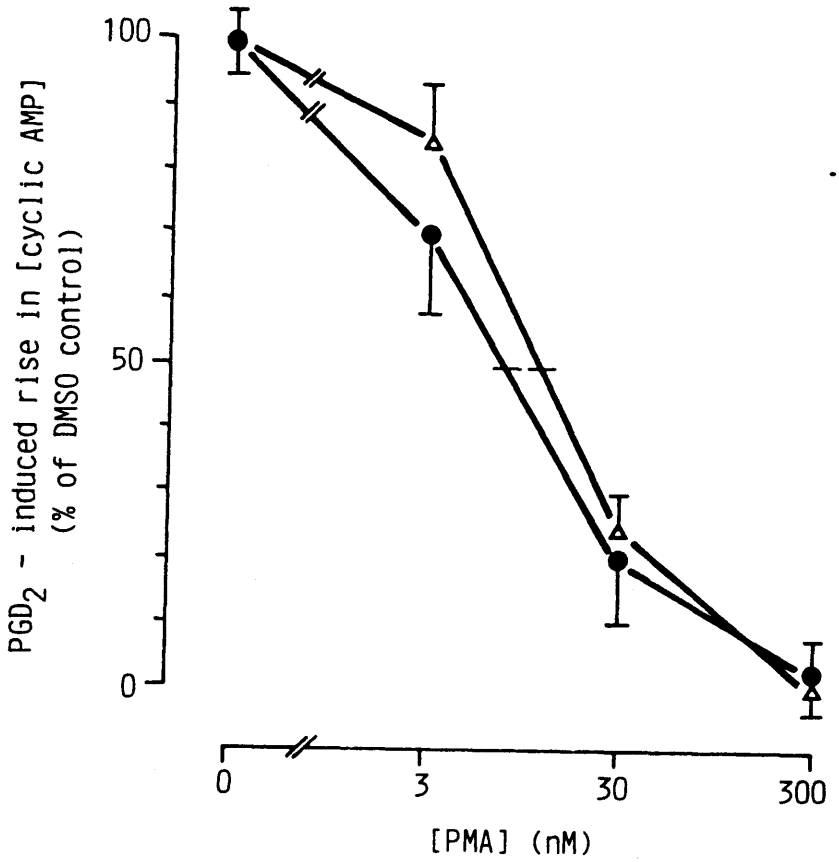

Figure 57. Effects of flurbiprofen on the inhibition of PGD_2-induced elevation of cyclic AMP content by PMA.

Control (●) and flurbiprofen (10 μM)-treated (△) gel-filtered platelet samples (0.4 ml) were incubated (37°C) for 2 minutes with PMA at the concentrations indicated before the addition of PGD_2 (300 nM) for a further 2 minutes and cyclic AMP content was monitored as described in Methods Section. For the sake of comparison between different experiments, results are expressed as percentage of the control rise in cyclic AMP content induced by PGD_2 in the presence of the PMA vehicle, DMSO (0.25%). The data are means and standard errors from three different experiments.

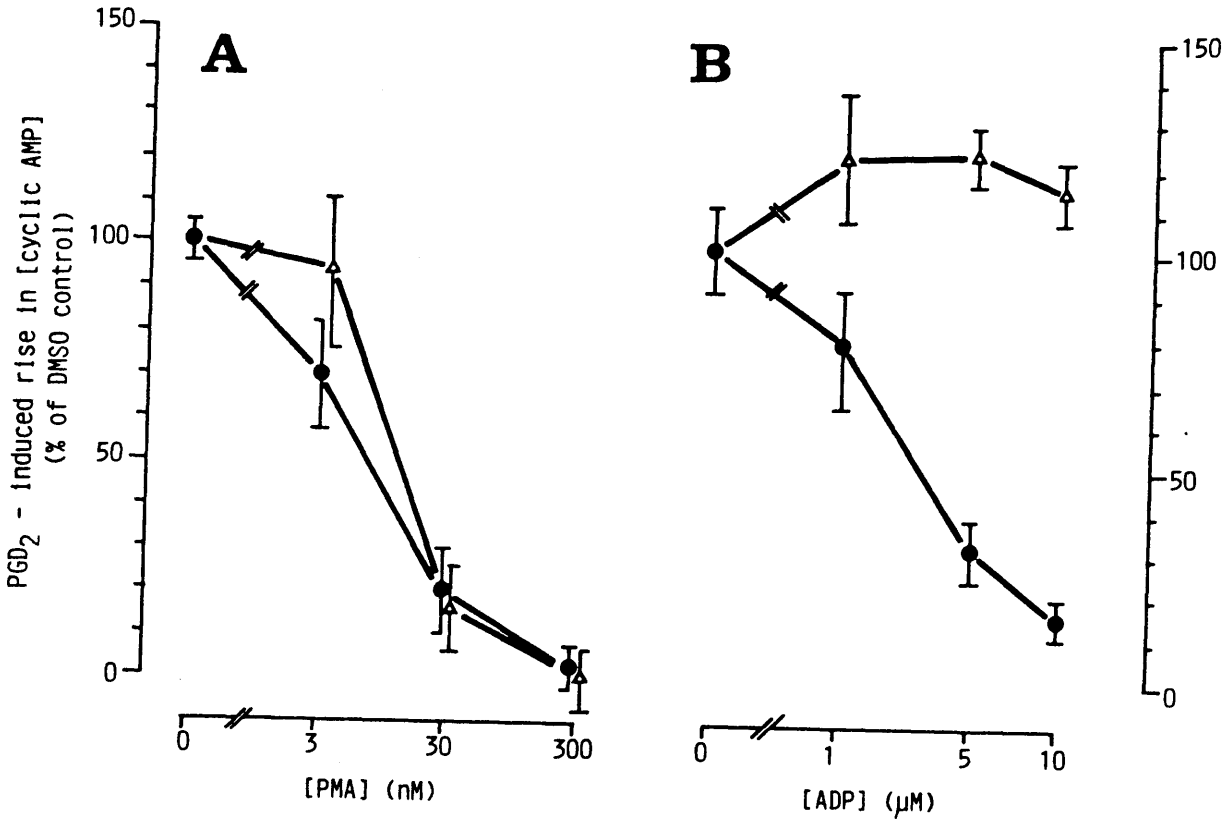

Figure 58. Effects of apyrase on the inhibition of PGD_2-induced elevation of cyclic AMP content by (A) PMA and (B) ADP.

Control (●) and apyrase (10 units of ADPase activity per ml; 10 min)-treated (△) gel-filtered platelet samples (0.4 ml) were incubated (37°C) for 2 minutes with (A) PMA or (B) ADP at the concentrations indicated before the addition of PGD_2 (300 nM) for a further 2 minutes. Cyclic AMP content was monitored as described in Methods Section. For the sake of comparison between different experiments, results are expressed as percentage of the control rise in cyclic AMP content induced by PGD_2 (300 nM) in the presence of the PMA vehicle, DMSO (0.25%). The data are means and standard errors from three different experiments.

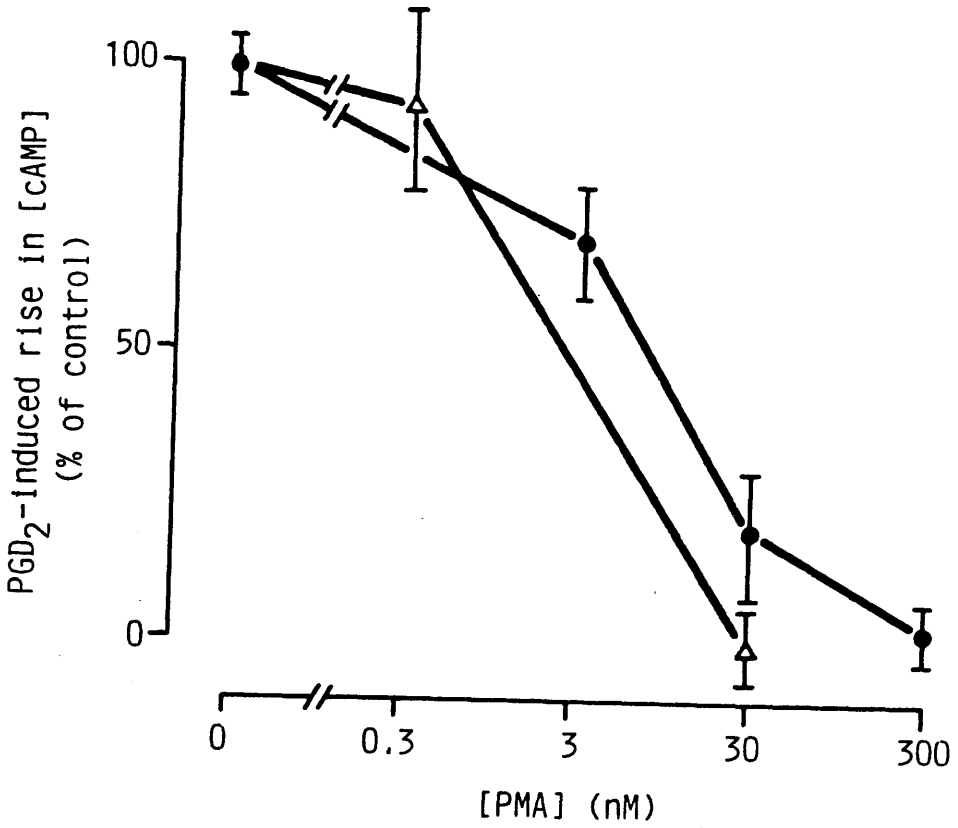

Figure 59. Effects of creatine phosphate/creatine phosphokinase on the inhibition of PGD_2-induced elevation of cyclic AMP content by PMA.

Control (●) and creatine phosphate (2 mM)/creatine phosphokinase (50 units of activity per ml; 10-40 min incubation)-treated gel-filtered platelet samples (△) were incubated (37°C) for 2 minutes with PMA at the concentrations indicated before the addition of PGD_2 (300 nM) for a further 2 minutes. Cyclic AMP content was monitored as described in Methods Section. For the sake of comparison between different experiments, results are expressed as percentage of the control rise in cyclic AMP content induced by PGD_2 in the presence of the PMA vehicle, DMSO (0.25%). The data are means and standard errors from three different experiments.

TABLE 6. Effect of IBMX on PGD_2-induced elevation of platelet cyclic AMP content.

Treatment	Cyclic AMP content (pmol/10^8 platelets)
Control	9 ± 0.6
PGD_2 (300 nM)	124 ± 10
PGD_2 (300nM) + IBMX (1mM)	231 ± 11

Control or IBMX (1 mM; 10-30 min; 37°C)-treated gel-filtered platelet samples (0.4 ml) were incubated (90 sec; 37°C) with saline or PGD_2 (300 nM) and cyclic AMP content was measured as described in Methods Section. Data are means and standard errors from three experiments using blood from different donors.

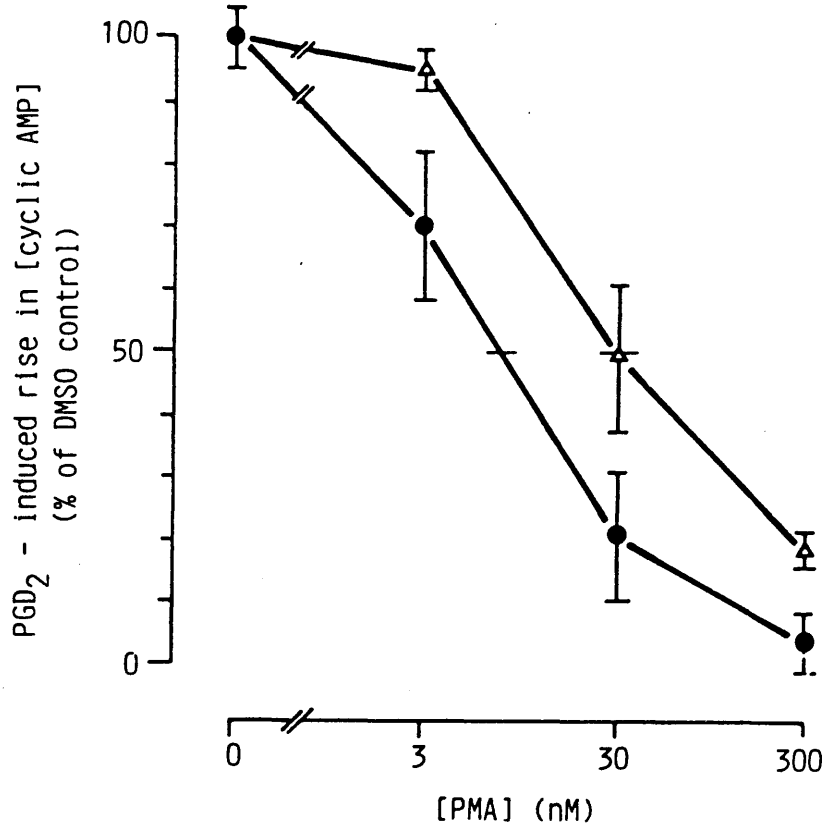

Figure 60. Effects of IBMX on the inhibition of PGD_2-induced elevation of cyclic AMP content by PMA.

Control (●) and IBMX (1 mM; 20-50 min)-treated gel-filtered platelet samples (Δ) were incubated (37°C) with PMA for 2 minutes at the concentrations indicated before the addition of PGD_2 (300 nM) for a further 2 minutes. Cyclic AMP content was monitored as described in Methods Section. For the sake of comparison between different experiments, results are expressed as percentage of the control rise in cyclic AMP content induced by PGD_2 in the presence of the PMA vehicle, DMSO (0.25%). The control rise in cyclic AMP was approximately doubled in the presence of IBMX (see Table 4). The data are means and standard errors from three different experiments.

V.2. Effects of PMA on elevation of cyclic AMP content induced by PGI_2, PGE_1, 6-keto-PGE_1, adenosine and reduction in cyclic AMP content induced by adrenaline

To investigate the specificity of this action, I next examined the effects of PMA on some other adenylate cyclase stimulants. Elevations in platelet cyclic AMP content induced by PGI_2 (100 nM), PGE_1 (1 μM) 6-keto-PGE_1, (1 μM) and adenosine, unlike PGD_2, were not significantly affected by pre-incubation (2 min: 37°C) with PMA, 300 nM (Figs. 61-63).

Possible effects of PMA on the ability of agonists (e.g. adrenaline) to inhibit adenylate cyclase were also assessed. Inhibition of PGI_2 (100 nM)-, PGD_2 (1 μM)- and PGE_1 (1 μM)-induced elevation in cyclic AMP content by adrenaline (5 μM; 30 sec pre-incubation) was unaffected by addition of PMA (300 nM) 90 sec before the adrenaline (Figs. 61 and 62).

VI. Protein kinase activity in crude platelet extracts

The aim of this study was to develop a simple assay for cyclic AMP-dependent protein kinase activity in crude platelet extracts which would allow the effects of platelet agonists and inhibitors on this enzyme to be determined.

VI.1. Effects of varying incubation time, platelet protein concentration and cyclic AMP concentration on kinase activity

Platelet protein kinase activity was stimulated by cyclic AMP (4 μM) and was linearly related to the incubation time at

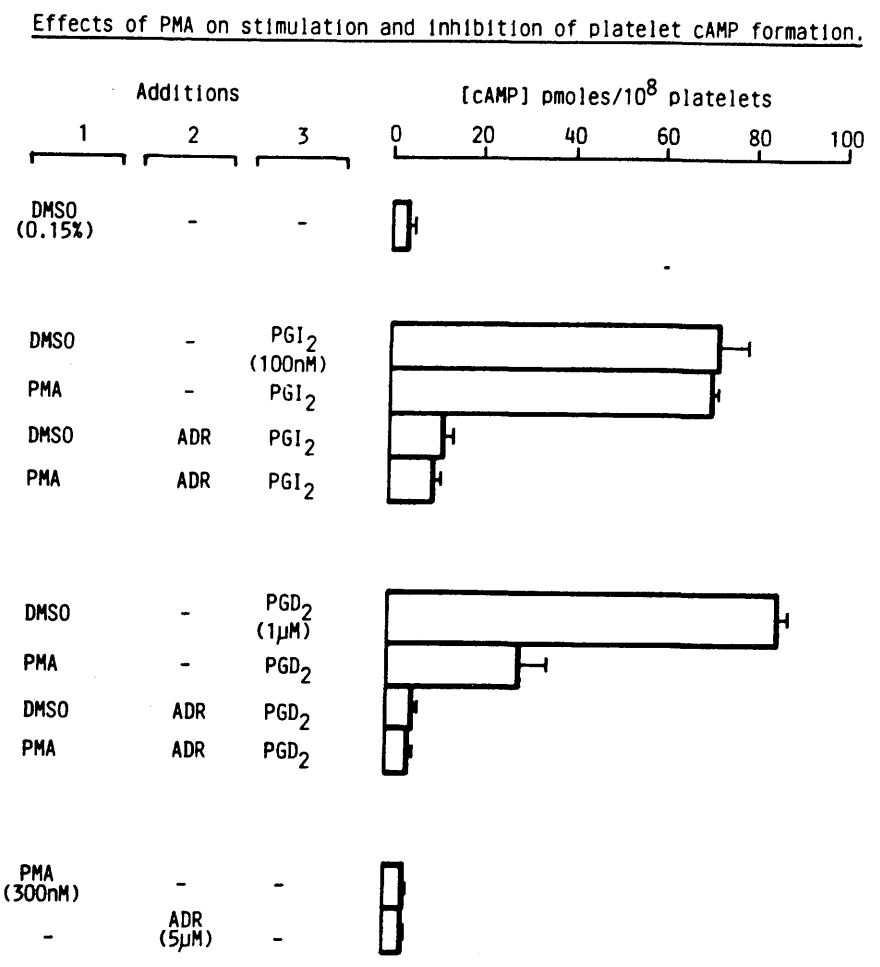

Figure 61. Effects of PMA on the modulation of platelet cyclic AMP levels by PGI_2 and adrenaline.

Where indicated, gel-filtered platelet samples (0.4 ml) were incubated ($37^{\circ}C$) with DMSO (0,15%; 4 min) or PMA (300 nM; 4 min) and/or adrenaline (5 μM; 2 min) and/or PGI_2 (100 nM; 1 min). Platelet cyclic AMP content was monitored as described in Methods Section. The data are means and standard errors from a single experiment representative of three.

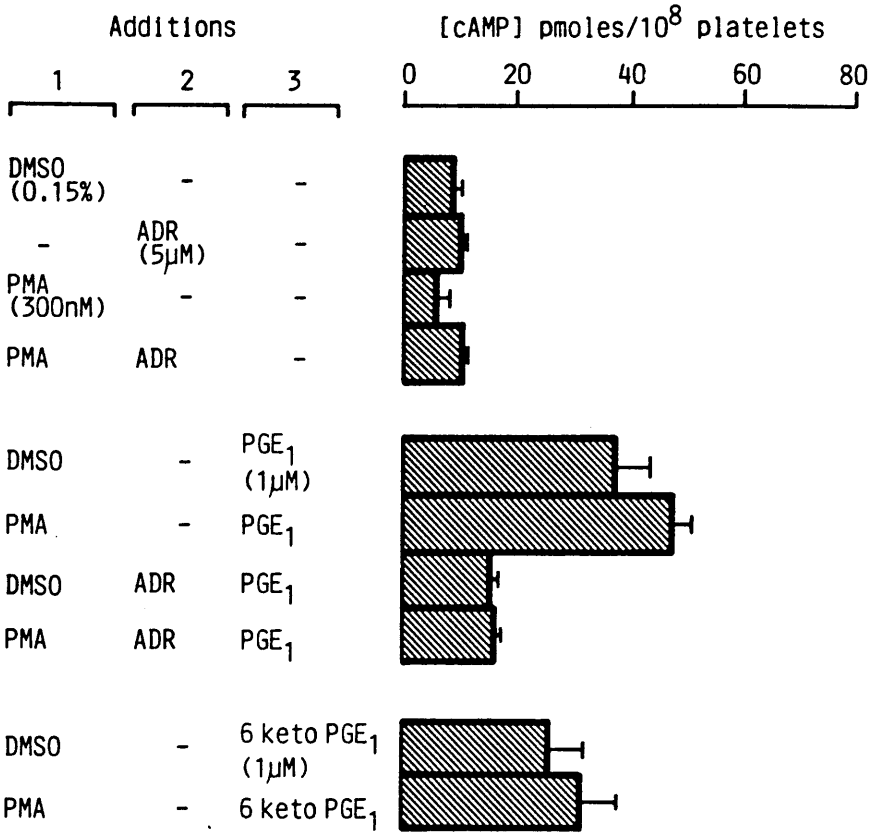

Figure 62. Effects of PMA on the modulation of platelet cyclic AMP levels by PGE_1, 6-keto-PGE_1 and adrenaline.

Where indicated, gel-filtered platelet samples (0.4 ml) were incubated ($37^{o}C$) with DMSO (0.15%; 4 min) or PMA (300 nM; 4 min) and/or adrenaline (5 μM; 2 min) and/or PGE_1 (1 μM; 1 min) or 6-keto-PGE_1 (1 μM; 1 min). Platelet cyclic AMP content was monitored as described in Methods Section. The data are means and standard errors from a single experiment representative of three.

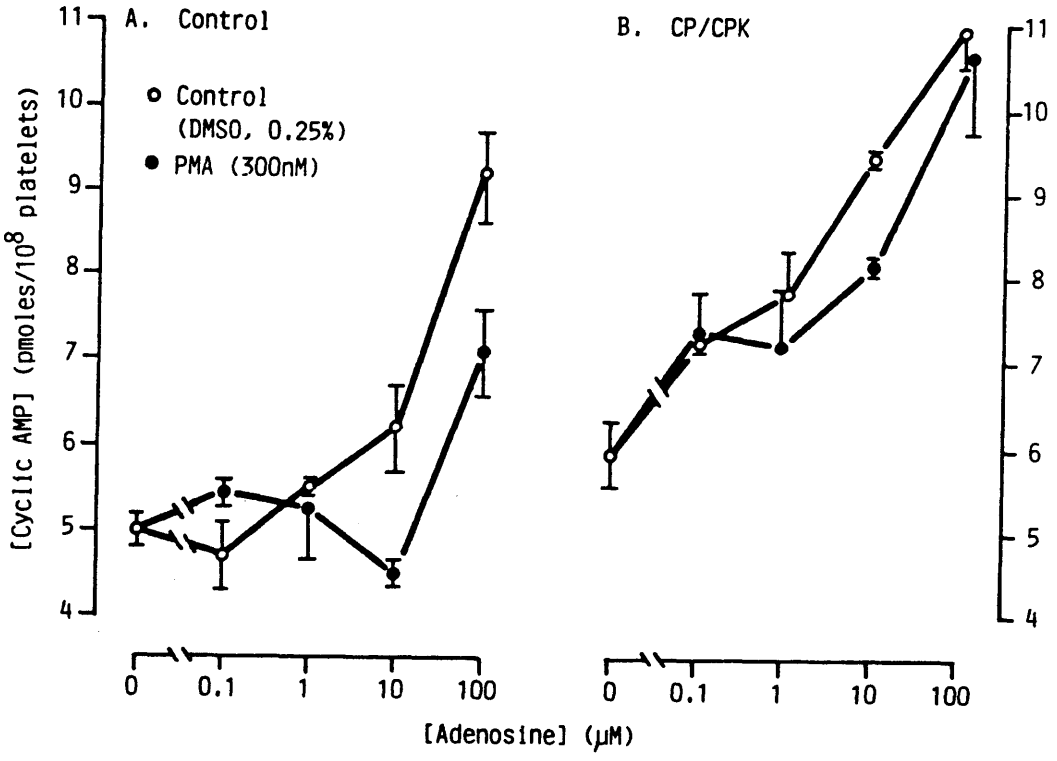

Figure 63. Effects of PMA (300 nM) on adenosine-induced elevation of platelet cyclic AMP content.

Control (A) and creatine phosphate (2 mM)/creatine phosphokinase (50 units per ml; 10 min incubation)-treated (B) gel-filtered platelet samples (0.4 ml) were incubated (2 min; 37°C) with PMA (300 nM) (●) or the vehicle (DMSO, 0.25%) (o) before addition of adenosine at the concentrations indicated for a further two minutes. Cyclic AMP content was measured as described in Methods Section. The data are means and standard errors from a single experiment representative of three similar.

30°C as shown in Figure 64. Platelet protein kinase activity also was linearly related to the concentration of platelet protein present in the assay mixture (Fig. 65). The protein concentrations normally used were 70-90 µg per assay of soluble protein and 20-30 µg per assay of particulate protein. Inclusion of increasing concentrations of cyclic AMP in the assay mixture resulted in concentration-dependent stimulation of kinase activity with an EC_{50} of around 0.56 µM cyclic AMP as shown in Figure 66 for soluble protein kinase activity.

VI.2. Effects of kinase and phosphatase inhibitors on platelet kinase activity

Inclusion of 25 units of the heat-stable inhibitor protein of cyclic AMP-dependent protein kinase (Ashby & Walsh, 1972) in the kinase assay mixture prevented stimulation of platelet protein kinase activity by cyclic AMP (4 µM) but had no significant effect on the basal enzyme activity (Fig. 67). Stimulation of soluble enzyme activity by cyclic AMP was also inhibited by the crude preparation of this inhibitor (25 units per assay) which was purchased from Sigma (Fig. 68). In contrast, inclusion of the calmodulin antagonist chlorprothixene (100 µM) and/or the combined calmodulin and protein kinase C antagonist trifluperazine (20 µM) (Sanchez et al., 1983) had no significant effect on soluble platelet protein kinase activity in the presence or absence of cyclic AMP, 5 µM (Fig. 68). Inclusion of NaF (40 mM) in the platelet lysis buffer (Buffer E) to inhibit phosphatase and ATP-ase activity

(Corbin et al., 1973) had no significant effect on basal or cyclic AMP (4 µM)-stimulated protein kinase activity (Table 7).

VI.3. Effects of stimulators of adenylate cyclase on platelet protein kinase activity

Incubation (2 min; 37°C) of platelet suspensions with PGI_2 (0.1 - 3 nM) or PGD_2 (3 - 300 nM) resulted in elevation of platelet cyclic AMP content but had no significant effect on platelet cyclic AMP-dependent protein kinase activity ratio (Table 8).

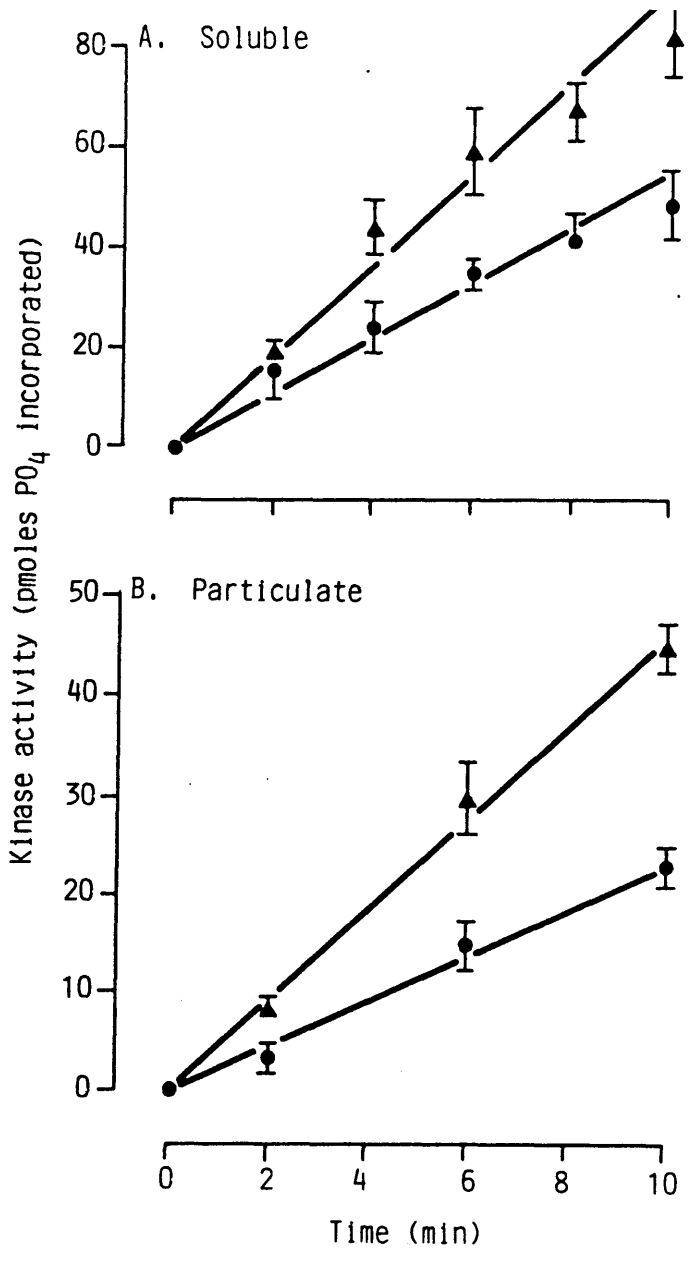

Figure 64. Effects of increasing incubation time on platelet protein kinase activity.

Platelet soluble (A) and particulate (B) fractions were incubated (30°C) for the times indicated in the presence (▲) and absence (●) of cyclic AMP (4 μM) and protein kinase activity was monitored as described in Methods Section. The data are means and standard errors from a single experiment representative of three.

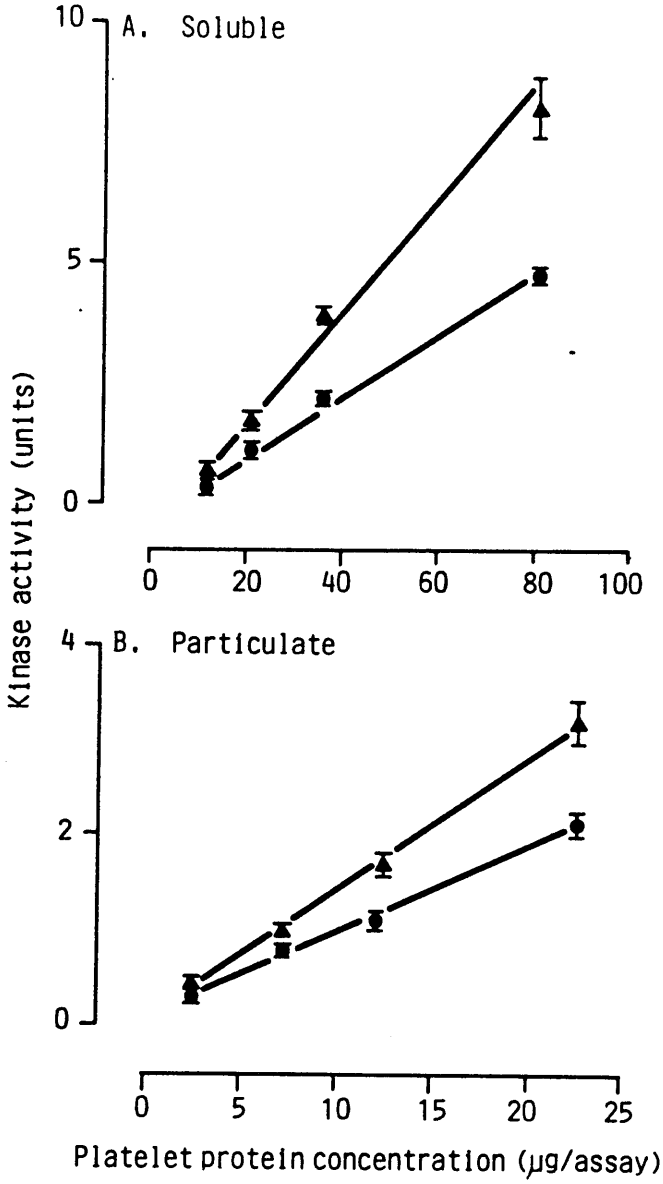

Figure 65. Effects of platelet protein concentration on kinase activity.

Platelet soluble (A) and particulate (B) protein fractions were incubated (10 min; 30°C) at the concentrations shown in the presence (▲) or absence (●) of cyclic AMP (4 µ M) for the measurement of protein kinase activity as described in Methods Section. The data are means and standard errors from a single experiment representative of two.

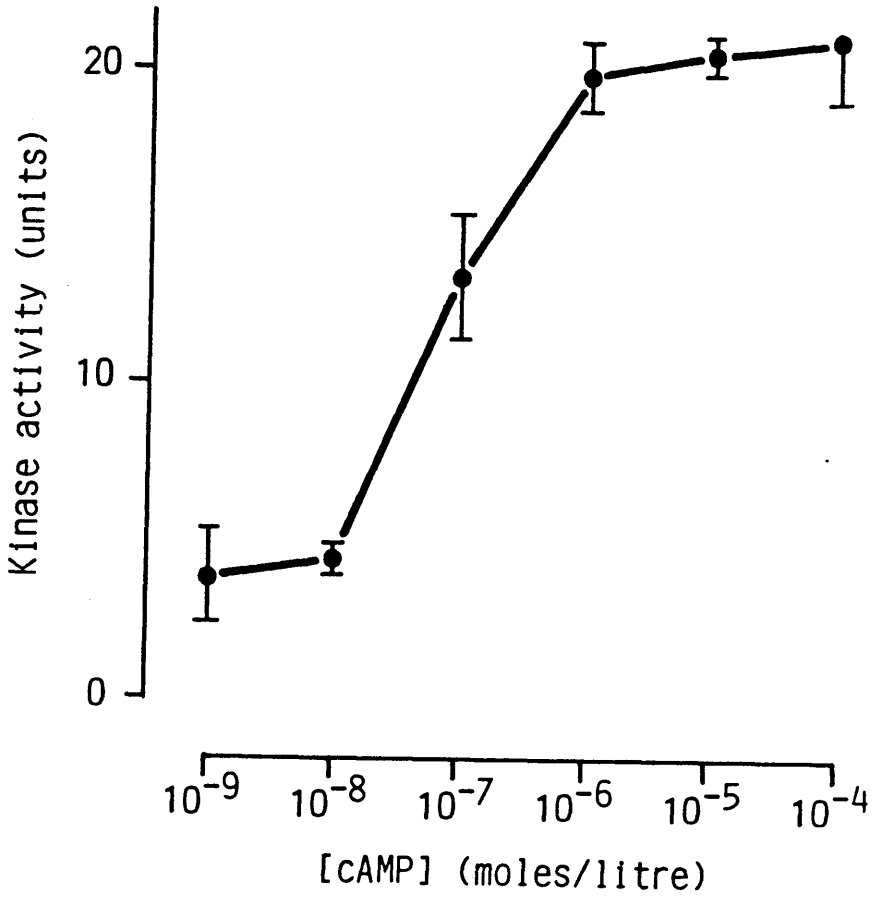

Figure 66. Effects of cyclic AMP on platelet soluble protein kinase activity.

Platelet soluble fractions were prepared and assayed for protein kinase activity in the presence of cyclic AMP at the concentrations indicated as described in Methods Section. The data are means and standard errors from a single experiment representative of two.

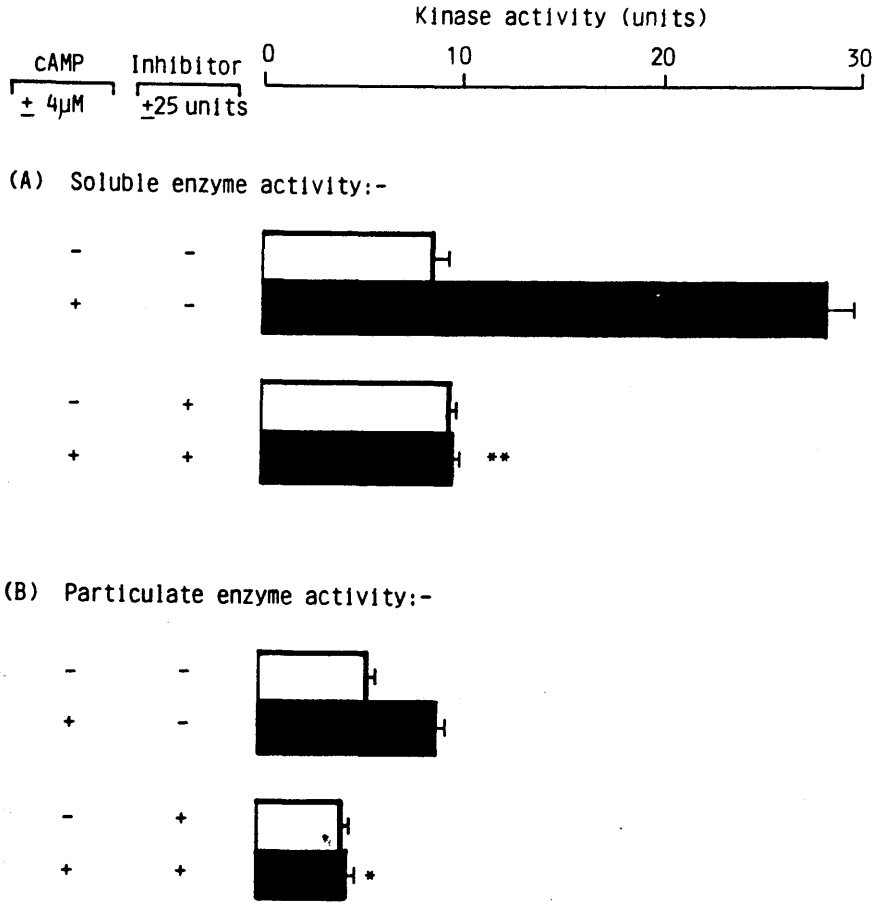

Figure 67. Effects of the inhibitor of cyclic AMP-dependent protein kinase on platelet protein kinase activity.

Platelet soluble (A) and particulate (B) fractions were prepared and assayed for protein kinase activity, as described in Methods Section, in the presence (where indicated) of cyclic AMP (4 μM) and/or the inhibitor of cyclic AMP-dependent protein kinase (25 inhibitory units per assay). The data are means and standard errors from a single experiment representative of three similar experiments. The data (presence versus absence of inhibitor) were compared using Student's t-test: *$P < 0.05$, **$P < 0.005$.

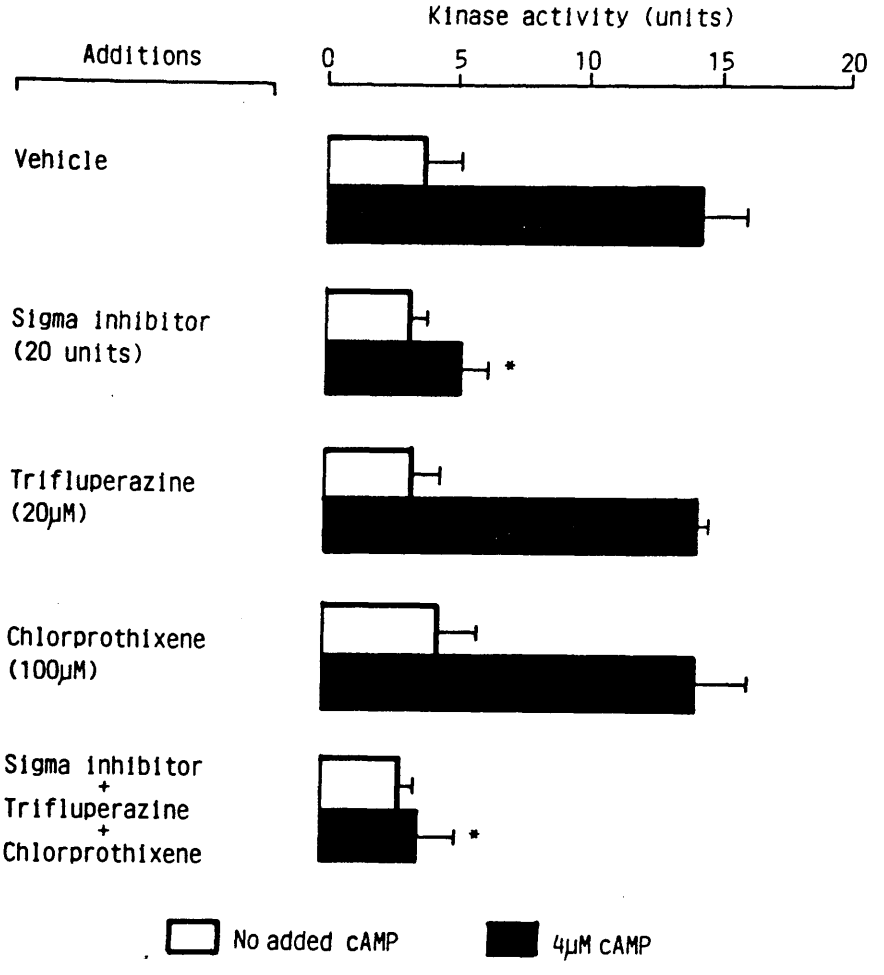

Figure 68. Effects of protein kinase inhibitors on platelet soluble protein kinase activity.

Platelet soluble fractions were prepared and assayed for protein kinase activity in the absence (open bars) or presence (hatched bars) of cyclic AMP (4 μM) and cyclic AMP-dependent protein kinase activity was monitored as described in Methods Section. Where indicated, the Sigma inhibitor of cyclic AMP-dependent protein kinase (20 inhibitory units per assay) and/or trifluperazine (20 μM) and/or chlorprothixene (100μM) were included in the assay mixture. The data are means and standard errors from a single experiment representative of two and were compared to controls (no inhibitors present) using Student's t-test: *$P < 0.05$.

TABLE 7. Effects of sodium fluoride on platelet soluble protein kinase activity.

	Protein kinase activity (units)		Activity Ratio
	-cAMP	+cAMP	
-NaF	13.8 ± 0.4	27 ± 0.7	0.51
+NaF	15.1 ± 1.3	27.3 ± 0.3	0.55

Platelet soluble fractions were prepared and assayed for protein kinase activity as described in Methods Section. The assay was performed in the absence and presence of cyclic AMP (4 μM) and NaF (40 mM). The data are means and standard errors from a single experiment representative of two.

TABLE 8. Effects of PGI_2 and PGD_2 on platelet protein kinase activity ratio.

		Soluble kinase (n)	Particulate kinase (n)
Control		0.50 ± 0.05 (18)	0.48 ± 0.07 (12)
PGI_2	0.1 nM	0.32 ± 0.05 (6)	0.35 ± 0.05 (3)
	0.3 nM	0.51 ± 0.08 (6)	0.34 ± 0.17 (3)
	3 nM	0.53 ± 0.09 (6)	0.40 ± 0.17 (3)
PGD_2	3 nM	0.52 ± 0.07 (7)	0.61 ± 0.11 (5)
	30 nM	0.57 ± 0.05 (7)	0.72 ± 0.19 (5)
	300 nM	0.66 ± 0.08 (7)	0.72 ± 0.08 (5)

Washed platelet suspensions (0.1 ml) were incubated (2 min; 37°C) with vehicle, PGI_2 or PGD_2 at the concentrations indicated, the reaction was terminated by freezing in solid CO_2/acetone and protein kinase activity ratio was determined as described in Methods Section. Data are means and standard errors from three experiments using blood from different donors.

DISCUSSION

The objective of this study was to examine the molecular mechanisms by which exogenous agents, and in particular combinations of agents, regulate platelet reactivity. To this end, the effects of agonists and agonist combinations on platelet functional responses and transduction processes were examined. This approach also allowed the interactions of the different second messenger systems in platelets to be examined. The possible functional relevance of the responses monitored using isolated platelets were assessed throughout this study by examining the responses of platelets in the more physiologically relevant milieu of whole blood. Due to the importance of platelets in haemostasis and thrombosis and the ease of preparation of an allegedly homogeneous population of platelets suspended in a buffer of defined composition, the study of platelet transduction processes is an extremely fast-moving research area. Hence, in the following discussion I shall attempt to place my results in perspective with respect to our current understanding of the molecular mechanisms of platelet activation and inhibition.

I. Measurement of platelet aggregation

In this study, aggregation was monitored in response to addition of adrenaline, ADP, vasopressin and PAF to whole blood, platelet-rich plasma or gel-filtered platelet suspensions. Photometric detection of aggregation in platelet-rich plasma or washed platelet suspensions, initially described by Born (1962), is the most commonly used technique for monitoring this response. A light beam is shone through a stirred platelet

sample (perpendicular to the axis of stirring) and transmitted light is detected by a photometer. An increase in light transmission is taken to reflect platelet aggregation. This technique, although immensely useful in the study of platelet physiology and pharmacology, has a number of inherent drawbacks, some of which were recognised as early as 1967. It is only semi-quantitative. Examination of ADP-induced aggregation directly, by light microscopic methods or electronic particle size analysis, indicates that extensive aggregation can occur in the absence of light transmission changes (Born & Hume, 1967; Nichols & Bosmann, 1979). Born & Hume (1967) reported 87% loss of single platelets with only 7% change in light transmission. A recent report has indicated that platelet aggregation can produce either decreases or increases in light transmission depending on the size and shape of the aggregate particles formed (Malinski & Nelsestuen, 1986). An additional complication in the analysis of light transmission data is the decrease in light transmission associated with shape change and increased platelet density (Michal & Born, 1971). Hence, quantitative analysis of aggregation traces, with respect to the absolute degree of aggregation present, is difficult.

In agreement with these findings, Figures 9-16 of this study show that adrenaline-, ADP-, vasopressin- and PAF-induced reductions in single platelet count occurred in response to lower agonist concentrations than were required for the induction of changes in light transmission in platelet-rich plasma. Changes in light transmission in response to higher agonist concentrations were preceded by reductions in platelet count (Figs. 9, 11, 13 and 15).

Photometric measurement of aggregation requires separation of platelets from other blood cells. Hence, aggregation in whole blood, a situation which more closely approximates the physiological milieu, cannot be examined photometrically. Since platelet reactivity may be modulated by agents released by other blood cell types, it would be advantageous to be able to correlate effects seen in platelet-rich plasma or washed platelets to responses in whole blood. Cardinal & Flower (1980) described an indirect method of monitoring platelet aggregation in whole blood which involved measuring the changes in electrical conductance through a blood sample which result from aggregate formation. In the present study, aggregation in whole blood was measured directly using the whole blood single platelet counting method of Lumley & Humphrey (1981). Using this method, adrenaline, ADP, vasopressin and PAF induced concentration-dependent aggregation in whole blood (Figs. 7, 8), indicating that these agonists could stimulate platelet activation in vivo. However, the concentrations of these agonists required to induce platelet aggregation in whole blood are at least an order of magnitude higher than those concentrations present in the circulation under physiological or pathological conditions (Grant & Scrutton, 1980a). Throughout this study, the physiological significance of responses (to agonists or agonist combinations) observed in platelet-rich plasma or isolated platelets was investigated by monitoring the responses in whole blood.

II. Calcium and inositol phospholipid hydrolysis in stimulus-response coupling

(a) PAF and vasopressin.

PAF and vasopressin induced $[^{32}P]$-Ptd OH formation and elevation of $[Ca^{2+}]_i$ over similar concentration ranges which induced platelet activation (Figs. 19-23). These findings are in agreement with other reports (MacIntyre & Pollock, 1983; Hallam et al., 1984a,b; Pollock & MacIntyre, 1986) and indicate that platelet receptors for PAF (paf_i) and vasopressin (V_i) are coupled to elevation of $[Ca^{2+}]_i$ and phosphoinositide hydrolysis. Kinetic analysis of agonist-induced elevations in $[Ca^{2+}]_i$ and phosphoinositide hydrolysis indicates that they precede the platelet responses and, hence, could act as transduction processes linking receptor occupancy to platelet activation (Fig. 21; MacIntyre et al., 1985c; Pollock & MacIntyre, 1986). The concentration-response relationships for PAF- and vasopressin-induced $[^{32}P]$-Ptd OH formation and elevation of $[Ca^{2+}]_i$ are superimposable (MacIntyre et al., 1985c; Pollock & MacIntyre, 1986); this is compatible with the theory that elevations in $[Ca^{2+}]_i$ are mediated by products of phosphoinositide hydrolysis (Berridge, 1985).

(b) ADP.

ADP induced aggregation and ATP secretion in washed platelet suspensions (Fig. 18) and this was associated with elevation of $[Ca^{2+}]_i$ (Figs. 21, 22) but not $[^{32}P]$-Ptd OH formation (Fig. 23). This lack of effect of ADP on phospho-inositide hydrolysis contrasts with studies using rat or

rabbit platelets (Vickers et al., 1982; MacIntyre & MacMillan, 1985) but is in agreement with another recent report using human platelets (Fisher et al., 1985). There are at least two possible explanations for these results. It is possible that ADP is a very weak activator of phosphoinositide hydrolysis in human platelets and that the technique used is not sensitive enough to detect small changes in Ptd OH formation. Alternatively, species variations may exist such that calcium fluxes can occur in human platelets in the absence of phosphoinositide hydrolysis. Indeed, Sage & Rink (1986) have recently reported that there are kinetic differences between the calcium fluxes induced by thrombin (a potent activator of phosphoinositide hydrolysis) and ADP. These authors suggested that thrombin- and ADP-induced calcium fluxes are mediated via distinct mechanisms. It is possible that human platelet ADP receptors could be directly coupled to opening of ROC (see introduction Section V). Receptor occupancy would then result in rapid influx of Ca^{2+} from the extracellular space in the absence of phosphoinositide hydrolysis or $Ins(1,4,5)P_3$ formation. The slower thrombin-induced elevation of $[Ca^{2+}]_i$ detected by Sage & Rink could be mediated by products of phosphoinositide hydrolysis.

(c) Adrenaline.

Adrenaline-induced ATP secretion and aggregation in washed platelet suspensions (Fig. 17) was apparently not associated with elevation of $[Ca^{2+}]_i$ (Figs. 21, 22) or phosphoinositide hydrolysis (Fig. 23). The lack of effect of adrenaline on platelet $[Ca^{2+}]_i$, monitored using Quin 2, contrasts with the reported adrenaline-induced changes in chlortetracycline

fluorescence (Le Breton et al., 1976), $^{45}Ca^{2+}$ uptake (Owen et al., 1980) and luminescence of platelets loaded with the calcium-sensitive photoprotein aequorin (Ware et al., 1986). These findings may indicate that adrenaline induces increases in $[Ca^{2+}]_i$ which are sufficiently small and/or sufficiently localised that it does not disturb the spatial average $[Ca^{2+}]_i$ which is reported by Quin 2 (Blinks et al., 1982; Johnston et al., 1985). The mechanism by which adrenaline might produce such a change in $[Ca^{2+}]_i$ is obscure since it is not accompanied by phosphoinositide hydrolysis, at least as monitored by measurement of $[^{32}P]$-Ptd OH formation (Fig. 23; MacIntyre et al., 1985c). Whether adrenaline-induced platelet activation could result from a decrease in the basal level of cyclic AMP, perhaps in some specific pool, is controversial and remains to be resolved (Salzman, 1972; Haslam et al., 1978; see below; Section VII).

III. Synergistic activation of platelets

III.1 Potentiation of platelet activation by adrenaline

Addition of low concentrations of adrenaline simultaneously with or up to 5 minutes before addition of ADP, vasopressin or PAF resulted in potentiation of aggregation monitored either in whole blood or gel-filtered platelets, and also potentiation of ATP secretion (Figs. 24-34). Potentiation occurred in the presence of the cyclooxygenase inhibitors aspirin (in whole blood) and flurbiprofen (in gel-filtered platelets) and therefore is not mediated via prostaglandin endoperoxide or TxA_2 formation (Figs. 24-34). These results

are in agreement with reports in platelet-rich plasma and washed platelets (Ardlie et al., 1966; Rao et al., 1980; Grant & Scrutton, 1980a; Vargaftig et al., 1982). It has recently been suggested that synergistic activation of platelets is not evident when aggregation is monitored by a single platelet counting method (Kerry & Scrutton, 1985). The results of this study (Figs. 24-31) indicate that this suggestion does not hold true, at least in whole blood. The finding of synergism in whole blood further supports the hypothesis that synergistic stimulation of platelet aggregation may also occur in vivo and may be of importance in haemostasis and in the pathogenesis of occlusive vascular disease.

III.2. Possible mechanisms of the potentiation of platelet activation by adrenaline

Since adrenaline and vasopressin utilise distinct transduction processes to evoke platelet activation, this combination was the major one, but not the only one chosen to examine synergism and to investigate the roles, if any, of phosphoinositide hydrolysis and elevation of $[Ca^{2+}]_i$ in potentiation of platelet activation by adrenaline. The results of this study demonstrate that the synergistic effects of adrenaline in combination with vasopressin, ADP or PAF are accompanied by enhancement of agonist-induced elevations in $[Ca^{2+}]_i$ (Fig. 35A-C) that derives partly from increased Ca^{2+} influx and partly from mobilisation of intracellular Ca^{2+}. This enhancement was unaffected by incubation with flurbiprofen and, therefore, is not mediated via prostanoid formation (Fig. 36). Adrenaline also potentiated vasopressin-induced phospho-

inositide hydrolysis (monitored as $[^{32}P]$-Ptd OH formation) (Fig. 37). The apparent dissociation between the temporal effects of adrenaline on vasopressin-induced phosphoinositide hydrolysis (Fig. 37) and elevation of $[Ca^{2+}]_i$ (Fig. 35A) may merely reflect the greater resolution of the assay of ^{32}P -Ptd OH, especially as the trend in $Ca^{2+}{}_i$ was always in the direction of an increase.

Even in the presence of adrenaline, agonist-induced elevations in $[Ca^{2+}]_i$ only attain levels of around 150-300 nM (i.e. some 50-200 nM above basal). At those concentrations $[Ca^{2+}]_i$, *per se*, is insufficient to account for the near-maximal stimulation of aggregation and ATP secretion observed under these circumstances (Rink *et al.*, 1982). This is confirmed by the observation that co-addition of vasopressin and ionomycin, at concentrations which elevated $[Ca^{2+}]_i$ to an extent comparable to or greater than that evoked by vasopressin and adrenaline in combination (Table 4), resulted in only slight enhancement of aggregation with no increment in ATP secretion (Fig. 38). Thus, potentiation of vasopressin-induced platelet activation by adrenaline cannot be attributed to enhanced elevation of $[Ca^{2+}]_i$ alone.

PMA mimics the effects of endogenous DG in activating protein kinase C and modulating platelet reactivity (Nishizuka, 1984). Incubation with PMA at subthreshold concentrations (at least for aggregation as measured photometrically) resulted only in marginal enhancement of vasopressin-induced platelet aggregation with no augmentation of ATP secretion (Fig. 38).

Thus, potentiation of vasopressin-induced platelet activation by adrenaline cannot be mimicked by an exogenous activator of protein kinase C and therefore, may not be attributable to enhanced DG formation alone.

In contrast, the effects of adrenaline on vasopressin-induced platelet aggregation and ATP secretion could be reproduced by subthreshold concentrations of PMA and ionomycin in combination (Fig. 38). Consequently, the mechanisms underlying potentiation of platelet activation by adrenaline may involve enhanced inositol phospholipid hydrolysis (ergo DG formation) and elevation of $[Ca^{2+}]_i$, perhaps consequent upon $Ins(1,4,5)P_3$ formation (Berridge, 1985).

These effects may be explained were adrenaline, either as a direct consequence of α_2-adrenoceptor occupancy or as a consequence of reduction in cyclic AMP concentration in a specific intracellular compartment, to induce a change in membrane lipid architecture (Irvine, 1982) or in a guanine nucleotide binding regulatory protein (Haslam & Davidson, 1984) and thus promote coupling of V_1 receptors to hydrolysis of PIP_2 via phospholipase C. Clearly, clarification of the roles of products of inositol phospholipid metabolism in the potentiation of platelet activation by adrenaline would require further investigation.

IV. Inhibition of platelet activation by cyclic AMP

IV.1 Inhibition of platelets in whole blood

Agonist-induced aggregation in whole blood was inhibited by the adenylate cyclase stimulants PGD_2 and PGI_2 (Figs. 39-42). This is in agreement with other studies using platelet-rich plasma and washed platelet suspensions (Di Minno et al., 1979; Feinstein et al., 1981). The degree of inhibition of ADP-induced aggregation was independent of the prostaglandin pre-incubation time (90 sec - 5 min) (Figs. 39, 40). Incubation (90 sec; 37°C) with PGD_2 (100 nM) or PGI_2 (3 nM) had little or no effect on adrenaline-induced aggregation but inhibited aggregation induced by ADP, vasopressin and PAF (Figs. 41, 42). Inhibition of the response to ADP, but not PAF or vasopressin, could be overcome by increasing the agonist concentration. This differential susceptibility to inhibition by prostaglandins is in agreement with a study in platelet-rich plasma (Di Minno et al., 1979) which demonstrated that higher concentrations of PGD_2, PGE_1 and PGI_2 were required to inhibit adrenaline compared to ADP or collagen.

It is widely accepted that these inhibitory prostaglandins act by raising the platelet intracellular cyclic AMP concentration (Feinstein et al., 1981). The receptors for adrenaline and ADP but not vasopressin or PAF can be coupled to inhibition of adenylate cyclase in intact platelets (Haslam & Rosson, 1975; Haslam & Vanderwel, 1982; Vanderwel et al., 1983; Aktories & Jakobs, 1985). Hence, adrenaline and ADP but not vasopressin or PAF can reverse prostaglandin-induced

elevations in platelet cyclic AMP content (Fig. 52). It seems likely that the inhibitory effects of adrenaline and ADP on platelet adenylate cyclase activity are responsible for the relative insensitivity of these agonists (compared to vasopressin and PAF) to inhibition by adenylate cyclase stimulants.

IV.2. Inhibition of different platelet functional responses and transduction processes by cyclic AMP

It has been proposed that elevated cyclic AMP concentrations inhibit platelet function by suppressing the production of the stimulatory second messenger molecules Ca^{2+} (Feinstein et al., 1983) and DG (Lapetina, 1984). This effect could be mediated by inhibition of phospholipase C and prevention of receptor-stimulated PIP_2 hydrolysis. A recent study by Halenda et al. (1986) demonstrated that elevation of cyclic AMP content by PGD_2 can interfere with the coupling of thrombin receptors to a GTP-binding protein in the platelet plasma membrane. This could be a general mechanism by which cyclic AMP inhibits agonist-induced phosphoinositide hydrolysis and elevation of $[Ca^{2+}]_i$. Adenylate cyclase stimulants cause a decrease in the elevated $[Ca^{2+}]_i$ when added after stimulatory agonists (Feinstein et al., 1983). This effect might be mediated by increased Ca^{2+} sequestration or extrusion (Feinstein et al., 1983; Kaser-Glanzmann et al., 1977); however, this seems incompatible with the reported lack of effect of cyclic AMP on ionophore-induced elevations in $[Ca^{2+}]_i$ (Pannochia &

Hardisty, 1985). In addition, cyclic AMP-dependent protein kinase has been shown to inhibit platelet myosin light chain kinase activity, a Ca^{2+}-activated enzyme (Hathaway et al., 1981). Hence, cyclic AMP can modify the rate of production and (possibly) inactivation of the stimulatory second messengers and can antagonise their effects to prevent or reverse agonist-induced platelet activation.

The results of this study show that PGD_2 was more potent as an inhibitor of PAF-induced ATP secretion (I_{50} around 2 nM) and aggregation (I_{50} around 3 nM) than of $[^{32}P]$-Ptd OH formation (I_{50} around 10 nM), shape change (I_{50} around 30 nM) and elevation of $[Ca^{2+}]_i$ (I_{50} around 30 nM) (Fig. 43). The degree of inhibitio of the various platelet responses could be correlated with the rise in cyclic AMP induced by PGD_2 in the same platelet samples (Figs. 44, 45). Small rises in cyclic AMP content (up to a 100% increase) induced by PGD_2 greatly inhibited PAF-induced aggregation and ATP secretion, with lesser inhibition of $[^{32}P]$-Ptd OH formation and only marginal inhibitions of shape change or elevation of $[Ca^{2+}]_i$. These results indicate that small rises in cyclic AMP content result in inhibition of some platelet functional responses (aggregation and secretion) but have relatively little effect on the putative underlying transduction processes: phosphoinositide hydrolysis and elevation of $[Ca^{2+}]_i$. These findings are consistent with cyclic AMP not only acting to prevent the formation or increase the inactivation of Ca^{2+} and DG but also acting at a later stage in the activation process to oppose the intracellular actions of Ca^{2+} and DG. These latter effects could be mediated

by inhibition of Ca^{2+}- or DG-dependent phosphorylation reactions or by inhibitory phosphorylation of the same target proteins that are acted upon by the 'stimulatory' kinases. There is some evidence that different protein kinases (Ca^{2+}-, DG- and cyclic AMP-dependent) share many phosphate acceptor proteins in vitro (Nishizuka, 1984). The observations reported here are in accord with a report by Pannochia & Hardisty (1985) in which PGI_2 and dibutyryl cyclic AMP were shown to inhibit platelet functions with little or no inhibition of the elevation of $[Ca^{2+}]_i$. It is concluded from this study that a major component of inhibition of PAF-induced platelet activatio by cyclic AMP is mediated by suppression of certain aspects of the activation process that occur distal to the formation or elevation of the stimulatory second messengers, Ca^{2+} and DG.

Clearly, PAF-induced shape change was much less susceptible to small rises in cyclic AMP content than were aggregation and ATP secretion (Figs. 43, 45). Similar results were found when PGI_2 was used as the inhibitory platelet agonist (Fig. 47). The reasons for this discrepancy are unknown, but it might suggest that the mechanisms underlying PAF-induced aggregation and secretion differ from those that govern shape change. It is possible that shape change is more dependent upon the absolute levels of the stimulatory second messengers, since inhibition of shape change parallelled inhibition of elevation of $[^{32}P]$-Ptd OH formation and $[Ca^{2+}]_i$. These findings are in disagreement with a report by Steen & Holmsen (1984) which demonstrated identical I_{50} values for cyclic AMP-dependent

inhibition of ADP-induced shape change, aggregation and ATP secretion. However, these studies may not be directly comparable, since both the stimulatory (ADP) and inhibitory (PGE_1 and adenosine) agonists used differed from those employed in the present study. Indeed, there is evidence that different agonists show differential sensitivity to inhibition by cyclic AMP (Krishnamurthi et al., 1984; Rink & Sanchez, 1984).

IV.3. Interactions between stimulators and inhibitors of adenylate cyclase

Platelets may be exposed, in vivo, to a combination of stimulants (e.g. PGI_2, PGD_2, adenosine) and inhibitors (e.g. circulating adrenaline or released ADP) of adenylate cyclase. Hence, in this study I examined the effects of combinations of stimulators and inhibitors of adenylate cyclase on agonist-induced platelet activation and the underlying transduction processes. The inhibition of PAF-induced aggregation in whole blood by PGD_2 or PGI_2 could be reversed by adrenaline (added after the PG) but not by vasopressin (Figs. 48, 49). Using washed platelet suspensions, adrenaline and ADP but not vasopressin reversed the inhibition of PAF-induced aggregation, ATP secretion and elevation of $\left[Ca^{2+}\right]_i$ which resulted from preincubation with PGI_2 (Fig. 50). Similarly, reversal of PGI_2-mediated inhibition of vasopressin-induced aggregation, ATP secretion, elevation of $\left[Ca^{2+}\right]_i$ and $\left[^{32}P\right]$-Ptd OH formation by adrenaline could also be demonstrated (Fig. 51). Under these conditions, PGI_2-induced elevations in platelet cyclic AMP content were prevented/reversed by adrenaline and ADP but

not vasopressin or PAF (Fig. 52). In contrast to this, elevations in platelet cyclic AMP content induced by the phosphodiesterase inhibitor IBMX (Table 5) and the inhibition of PAF-induced platelet responses by IBMX or 8 bromocyclic AMP were not significantly affected by adrenaline (Figs. 53, 54). Hence, elevation of cyclic AMP levels and inhibition of platelet responsiveness by adenylate cyclase stimulants, but not by other mechanisms, can be prevented or reversed by inhibitors of adenylate cyclase (adrenaline and ADP) but not by other platelet agonists (vasopressin and PAF). These results are in agreement with those of Zavoico & Feinstein (1984) who showed that inhibition of thrombin-induced elevation of $[Ca^{2+}]_i$ and secretion by pre-incubation with adenylate cyclase stimulants could be prevented by adrenaline or ADP. This effect was evident irrespective of whether adrenaline was added before or after the adenylate cyclase stimulant (PGI_2, PGD_2 or forskolin) or after the thrombin (Zavoico & Feinstein, 1983).

These findings confirm the previous reports that cyclic AMP regulates the stimulatory transduction processes of elevation of $[Ca^{2+}]_i$ and phosphoinositide hydrolysis (Feinstein *et al.*, 1983; Lapetina, 1984). In the presence of multiple platelet agonists, the net effects of the various receptors on the guanine nucleotide-binding regulatory proteins N_s and N_i will control the rate of cyclic AMP formation. Hence, *in vivo*, the dynamic balance between stimulation and inhibition of adenylate cyclase will control the platelet response i.e. inhibition or stimulation. This could be an important regulatory mechanism in the control of haemostasis.

V. Effects of protein kinase C activation on platelet cyclic AMP metabolism

A number of recent studies have demonstrated that protein kinase C, besides mediating platelet activation, can also modulate receptor-controlled transduction processes. Protein kinase C can stimulate receptor phosphorylation in a number of different cell types (Sibley et al., 1984). In addition, activated protein kinase C can inhibit agonist-induced phosphoinositide hydrolysis and elevation of $[Ca^{2+}]_i$ in platelets (MacIntyre et al., 1985d) and other cells (Drummond & MacIntyre, 1985). This effect may be explained by the finding that, in platelets, protein kinase C activation interferes with the interaction of stimulatory receptors (e.g. thrombin) with a GTP-binding protein (Halenda et al., 1986). It has also been suggested that one of the '40K' proteins phosphorylated by protein kinase C is an $Ins(1,4,5)P_3$ phosphatase (Connolly & Majerus, 1986). This could also contribute to the inhibition of Ca^{2+} mobilisation by activators of protein kinase C (MacIntyre et al., 1985c,d).

Studies using tumour-promoting phorbol esters such as PMA have demonstrated that activated protein kinase C can modulate cyclic AMP metabolism in a number of different cell types. Basal cyclic AMP levels may be increased (Grotendorst & Schimmel, 1980; Brostrom et al., 1983; Cronin and Canonico, 1985; Quilliam et al., 1985), decreased (Brostrom et al., 1982) or unaffected (Pick et al., 1977; Belman & Garte, 1980) after treatment with phorbol esters. Similarly, differential

effects of phorbol esters on elevated cyclic AMP levels have been reported (see references above). In this study, I examined the effects of phorbol esters on platelet cyclic AMP metabolism. PMA, at concentrations and incubation times known to activate protein kinase C (Castagna et al., 1982), had no effect on control or IBMX-elevated platelet cyclic AMP content (Fig. 55).

Inhibition of PGD_2-induced elevation of platelet cyclic AMP content by PMA was not mimicked by the non-tumour-promoting phorbol ester 4α-PDD (Fig. 56) suggesting that this effect of PMA is due to activation of protein kinase C rather than any non-specific membrane perturbation caused by the detergent-like nature of the phorbol esters (Castagna et al., 1982). The effect of PMA was not mediated by the liberation of endogenous ADP from the dense granules: inhibition of the PGD_2-induced elevation in cyclic AMP occurred in the presence of ADP-removal systems (apyrase and CP/CPK) (Figs. 58, 59). This effect of PMA also occurred in the presence of an inhibitor of cyclooxygenase (flurbiprofen) and, therefore, is not mediated via prostanoid biosynthesis (Fig. 57). Incubation with IBMX resulted in only marginal inhibition of the effect of PMA suggesting that it is not mediated by increased phosphodiesterase activity (Fig. 60). Taken together, these results indicate that activation of protein kinase C by PMA directly interferes with PGD_2-induced cyclic AMP formation. Possible mechanisms of this action include (1) inactivation of the DP receptor for PGD_2; (2) inactivation of the stimulatory G-protein N_s; (3) activation of the inhibitory G-protein N_i

or (4) inactivation of the adenylate cyclase catalytic subunit (Fig. 2).

Elevations in cyclic AMP content induced by adenylate cyclase stimulants which act via IP receptors (PGI_2, PGE_1, 6-keto-PGE_1) and A_2-purinoreceptors (adenosine) were unaffected by PMA (Figs. 61-63). The specificity of this effect of PMA suggests that it is mediated via an action at the DP receptor rather than the G-proteins or the adenylate cyclase catalytic subunit which are presumably common to different agonists (Fig. 69). In this context, it would be of interest to examine in more detail the nature of the inhibition of PGD_2 by PMA and the effects of PMA on the numbers and affinity of platelet DP, IP and A_2 receptors. Ashby et al. (1985) reported that PMA or the synthetic DG analogue, 1-oleoyl-2-acetyl glycerol (OAG), had no effect on PGI_2- or forskolin-induced cyclic AMP formation in platelets. This confirms the lack of any direct effect of protein kinase C on the catalytic subunit of adenylate cyclase. Jakobs et al. (1985) reported that forskolin- and PGI_2-induced stimulation of adenylate cyclase activity in platelet membrane preparations was slightly enhanced by pre-treatment of the platelets with PMA.

It is perhaps worthy of note here that PGD_2, unlike the other inhibitory agonists studied, can be produced by stimulated platelets (Hamberg & Fredholm, 1976). The specificity of this effect of PMA may suggest that activation of protein kinase C in platelets could provide a regulatory mechanism to abrogate the effects of the endogenous adenylate cyclase stimulant

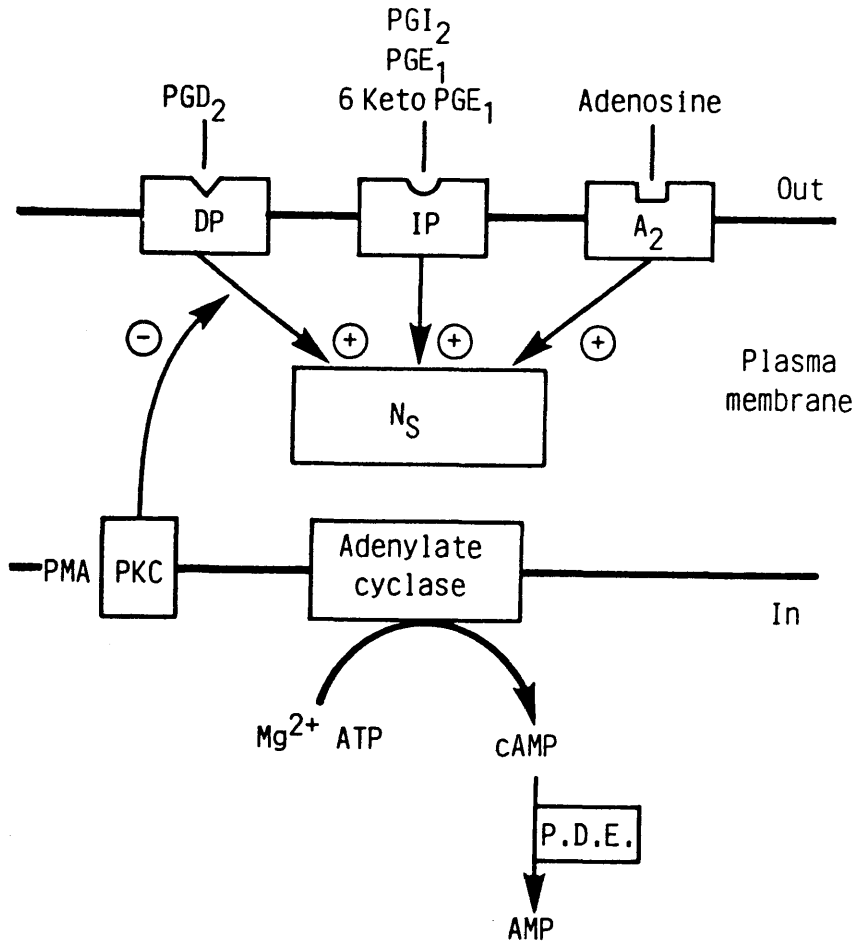

Figure 69. Scheme depicting the effects of PMA on receptor-controlled cyclic AMP metabolism in platelets.

Activation of protein kinase C by PMA inhibits DP- but not IP- or A_2-receptor-mediated cyclic AMP formation. See text for details.

(PGD_2) without compromising the actions of exogenous stimulants of adenylate cyclase such as endothelium-derived PGI_2. However, the possible physiological or pathological importance of this phenomenon in the regulation of platelet reactivity remains to be ascertained.

In intact platelets, PMA had no effect on the inhibition of PGD_2-, PGI_2-, PGE_1- or 6-keto-PGE_1-induced elevation of cyclic AMP content by adrenaline (Figs. 61, 62). However, Jakobs et al. (1985) reported that PMA pretreatment abolished N_i-mediated inhibition of adenylate cyclase by adrenaline in platelet membranes. It was proposed that this effect of PMA was mediated by protein kinase C-catalysed phosphorylation of N_i (Watanabe et al., 1985; Katada et al., 1985). The reasons for this discrepancy are unknown, however, it does suggest that studies using platelet lysates do not always reflect the situation in intact cells where there may be complex interactions between the different second messenger systems. This is confirmed by a report by Murayami & Ui (1985) who found that the coupling of thrombin receptors on mouse 3T3 fibroblasts to N_i and inhibition of adenylate cyclase was attenuated by cell disruption and preparation of membrane fractions.

VI. Platelet cyclic AMP-dependent protein kinase activity

The object of this study was to develop a simple, reproducible assay for cyclic AMP-dependent protein kinase activity in crude platelet extracts and to examine the effects of platelet agonists on this enzyme. Since kinase activity is coupled very closely to changes in cyclic AMP concentration in a range of cell and tissue types, measurement of the activity of this enzyme provides a very sensitive index of small changes in intracellular cyclic AMP concentration (Corbin, 1983). It was hoped that development of such an assay in platelets might help resolve the contentious issue of the possible involvement of reductions in the basal level of cyclic AMP, perhaps in some specific intracellular pool, in the activation of platelets by stimulatory agonists, particularly adrenaline (Salzman, 1972; Haslam et al., 1978; Jakobs et al., 1986). Although stimulatory agonists do not have any effect on the basal platelet cyclic AMP content (Haslam et al., 1978), there is some evidence that agonists can alter specific pools of cyclic AMP in rabbit platelets (Hashimoto, 1983). There is also evidence of compartmentalisation of cyclic AMP binding proteins in platelets (Lyons, 1980) and other cells (Buxton & Brunton, 1983).

In agreement with previous reports (Salama & Haslam, 1981; 1984) kinase activity was present in both soluble and particulate platelet fractions. The degree of kinase activity detected varied linearly with incubation time (Fig. 64) and platelet protein concentration (Fig. 65) and was stimulated

in a concentration-dependent manner by cyclic AMP (Fig. 66). Activity in the presence, but not the absence, of added cyclic AMP was inhibited by the specific heat-stable inhibitor of cyclic AMP-dependent protein kinase (Ashby & Walsh, 1972) but not by inhibitors of calmodulin (chlorprothixene and trifluperazine) and protein kinase C (trifluperazine) (Fig. 67, 68). These results indicate that I was able to detect the activity of a functional cyclic AMP-dependent protein kinase enzyme extracted from platelets and that this resembled (functionally) the cyclic AMP-dependent protein kinase of other tissues (Corbin, 1983).

However, I was unable to detect any reproducible stimulation of this enzyme in platelets treated with the adenylate cyclase stimulants PGI_2 and PGD_2 (Table 8). There are a number of possible explanations for this discrepancy including (1) artificial dissociation of the regulatory, R, and catalytic, C, subunits of the enzyme during platelet lysis; (2) artificial reassociation of R and C; (3) binding of C to particles other than R; (4) inhibition of kinase by salts present in the assay mixture; (5) presence of proteases and (6) release of sequestered cyclic AMP during platelet lysis (reviewed by Corbin, 1983). These problems would have to be resolved before the possible effects of stimulatory agonists, such as adrenaline, could be assessed. I believe that given enough time, these possible pitfalls could be systematically evaluated and the technical difficulties of this assay could be overcome. This might yield important new insights into the mechanisms by which agonists, particularly adrenaline, control platelet function.

Another possible approach to the problem of detecting very small changes in cyclic AMP levels is to measure the functional pool of cyclic AMP which is bound to the R subunit of cyclic AMP-dependent protein kinase. This approach has been used successfully in adrenocortical cells (Podesta *et al.*, 1979) in order to demonstrate the role of small changes in cyclic AMP concentration in the stimulation of corticosterone production by low concentrations of corticotropin. This assay technique might profitably be applied to platelets.

In conclusion, this study has addressed several questions in relation to the mechanisms by which agonists and agonist combinations control platelet reactivity. Some of these questions have been answered whilst several others have been generated. Many aspects of the study are incomplete and might fruitfully be pursued further. In particular, further investigation would be required to elucidate fully the mechanisms underlying potentiation and synergistic stimulation of platelet reactivity. Platelets could be exposed simultaneously or sequentially to several different agonists *in vivo*. Therefore, it would be of interest to examine the effects of co-addition of multiple stimulatory agonists (as opposed to agonist pairs) on platelet reactivity and the mechanisms which mediate these effects. Detailed understanding of the mechanisms underlying synergism might facilitate the rational development and selection of agents which impinge upon the processes involved in potentiation. Such agents may more effectively inhibit platelet reactivity in whole blood *in vivo*, and could be of benefit in the prevention and treatment of occlusive vascular disease.

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