DESENSITISATION AND POTENTIATION OF PLATELET INOSITOL PHOSPHOLIPID HYDROLYSIS

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ABBREVIATIONS

The abbreviations used in this thesis are those recognised by the Biochemical Journal. The more commonly used abbrevations include:-

Ptd Ins	phosphatidylinositol
Ptd OH	phosphatidic acid
PIP	phosphatidylinositol 4 phosphate
PIP_2	phosphatidylinositol 4,5 bisphosphate
$[Ca^{2+}]_i$	cytosolic free calcium concentration
Quin 2	2-methyl-6-methoxy-8-nitroquinoline
5HT	5 hydroxytryptamine; serotonin
ADP	adenosine 5' diphosphate
ATP	adenosine 5' triphosphate
PAF	platelet activating factor
Tx	thromboxane
PG	prostaglandin
DAG	1,2 diacylglycerol
$ins(1,4,5)P_3$	inositol 1,4,5 trisphosphate
cAMP	adenosine 3'5' cyclic phosphate
cGMP	guanosine 3'5' cyclic phosphate
PMA	phorbol 12-myristate 13-acetate
OAG	1-oleoyl 2-acetyl glycerol
Kd	dissociation constant
Ptd Ser	phosphatidyl serine
Ptd Cho	phosphatidyl choline
8Br cAMP	8-bromoadenosine 3'5' cyclic monophosphate
8Br cGMP	8-bromoguanosine 3'5' cyclic monophosphate
NaNP	sodium nitroprusside
GDW	glass distilled water

SUMMARY

Human platelet responsiveness can be modulated (stimulated or inhibited) by a variety of stimuli, many of which act at specific receptors on the external surface of the plasma membrane The mechanisms by which this external stimulus is transferred to the final cellular response are termed generically "stimulus response coupling" and invariably involve the production of second messenger molecules which govern the final cellular response. In platelets, and some other cells, stimulation is believed to be associated with, and mediated by, the production of two second messengers, 1,2 diacylglycerol (DAG) and inositol 1,4,5 trisphosphate ($Ins(1,4,5)P_3$). Agonist receptor interaction results in the phospholipase C catalysed hydrolysis of phosphatidylinositol 4,5 bisphosphate (PIP_2) to yield DAG and $Ins(1,4,5)P_3$ directly. $Ins(1,4,5)P_3$ mobilises calcium from an intracellular store, and this elevated cytosolic calcium level activates a Ca-calmodulin dependent protein kinase which, in turn, catalyses the phosphorylation of a protein of M_r = 20,000 which plays an integral role in the stimulation of platelets. At resting calcium levels, DAG translocates a soluble cytosolic enzyme, protein kinase C, to the membrane and subsequently activates it. Protein kinase C in turn phosphorylates a protein with a M_r of 40,000, which also has been implicated in platelet function and which may act independently of, or synergistically with, the calcium mediated activation. In contrast, platelets are maintained in a quiescent state by agents which cause an elevation in the intracellular concentration of either cAMP

or cGMP. These two inhibitory platelet mediators presumably activate specific kinases which phosphorylate proteins which function to maintain the quiescent state.

Cellular reactivity is the subject of control by a number of factors, apparently dependent upon extracellular conditions. For example numerous exogenous agonists promote platelet activation whereas endogenous compounds such as PGI_2 and PGD_2 inhibit platelet reactivity. Platelets elicit the phenomenon of desensitisation whereby the addition of an agonist renders them less responsive to agonists which act on the same receptor (homologous desensitisation) or to agonists that act upon distinct receptors (heterologous desensitisation). In contrast under some conditions, combinations of agonists can produce a response which is larger than that evoked by either agonist alone (potentiation).

I attempted to clarify the role of receptor stimulated phosphoinositide hydrolysis in mediating the activity of a number of agonists whose putative mechanism of action was unknown or controversial. Phosphoinositide hydrolysis was routinely monitored by measuring the formation of $[^{32}P]$ phosphatidic acid (Ptd OH) in platelets prelabelled with $[^{32}P]$ orthophosphate. Ptd OH is the phosphorylated product of DAG, and the experimental conditions adopted allowed $[^{32}P]$ Ptd OH to obtain isotopic equilibrium with gamma- $[^{32}P]$ ATP such that changes in label reflect changes in Ptd OH content. In addition in some studies depletion of $[^{32}P]$ PIP_2 was monitored.

Cytosolic free calcium concentration was measured by Quin 2. In addition, I evaluated the role of various putative endogenous mediators of desensitisation on the stimulatory transduction process(es) and addressed the possible mechanism(s) whereby agonist-induced platelet reactivity could be potentiated.

The major findings are outlined below:-

1) ADP failed either to elicit the formation of $[^{32}P]$ Ptd OH or to cause the degradation of $[^{32}P]$ PIP_2.

2) The stable thromboxane A_2 analogue, EP171, elicits a concentration dependent secretion of ATP, formation of $[^{32}P]$ Ptd OH and also depletion of $[^{32}P]$ PIP_2. The effects of EP171 on ATP secretion and $[^{32}P]$ Ptd OH formation are attenuated by U44069 and Et PGH_2.

3) Thrombin stimulated both $[^{32}P]$ Ptd OH formation and $[^{32}P]$ PIP_2 hydrolysis. In both respects thrombin is the most efficacious of all agonists of potential patho-physiological importance (PAF, 5HT, TxA_2 (ergo EP171 or U44069), vasopressin).

4) Trypsin stimulates elevated levels of $[^{32}P]$ Ptd OH.

5) EP171 in combination with ADP, 5HT and PAF did not synergise in the formation of $[^{32}P]$ Ptd OH, whereas the combination of EP171 and trypsin did produce additive effects.

6) PGD_2, sodium nitroprusside and phorbol ester activators of protein kinase C all inhibit and reverse an agonist induced elevation in $[Ca^{2+}]_i$ and $[^{32}P]$ Ptd OH formation but do not affect the resting levels of either.

7) Adrenaline and ADP, neither of which stimulate the formation of $[^{32}P]$ Ptd OH, both potentiate agonist-induced formation of $[^{32}P]$ Ptd OH. This effect is opposed by agents which stimulate adenylate cyclase.

These results indicate that thrombin and TxA_2 (ergo EP171) but not ADP induced stimulation of platelets is mediated by phospholipase C activation. Thrombin is the most efficacious platelet agonist and this may reflect stimulation via both a receptor-mediated and proteolytic manner. U44069, like Et PGH_2, is a partial agonist at the platelet Tx receptor. Adenylate cyclase, guanylate cyclase and protein kinase C stimulants all inhibit receptor mediated production of stimulatory second messengers. Therefore cAMP, cGMP and/or DAG may act as endogenous mediators of desensitisation. Adrenaline and ADP induced potentiation of platelet reactivity may be due to an effect at or prior to phospholipase C.

INTRODUCTION

CHAPTER I: THE PLATELET

1. General

The observations of Bizzozero and of Hayem, both in 1882, demonstrated the presence of small elements in circulating blood which adhered initially to damaged vascular walls and subsequently to each other to form haemostatic plugs. Prior to these initial reports of the existence of platelets, their presence had been neglected by microscopists due to the small size of the cell in comparison to the larger red and white cells. The proposal that platelets were artifacts of inadequate blood preparation (Buckmaster, 1906) was quickly dispelled by much work carried out in the early 20th century. Although the majority of these early studies were concerned with the role of the platelet in coagulation, it soon became evident that platelets had a wide range of physiological and pathological functions. In addition platelets are widely used as a model system for studying other processes due, at least in part, to their ease of availability at little cost, the facility with which a homogenous population of cells can be obtained and the multiplicity of receptors which they possess.

2. Formation of platelets

The origin of the platelet was identified by Wright in 1906 as being the megakaryocyte. It is believed that the megakaryocyte shares a common precursor, or pluripotential stem cell, with myeloid and erythroid cells (Becker *et al.*, 1963). Once these cells become committed to the production of megakaryocytes, they undergo three stages of maturation

during each of which there is continual cell growth (Levin et al., 1981).

Firstly, the megakaryoblast stage, during which the cell initially can be identified under a light microscope (Paulus, 1970). There is intense protein synthesis at least part of which can be accounted for by the formation of specific membranes These membranes become internalised and this, when combined with the endomitosis that occurs, may represent a specialised form of the intracellular development of daughter cells (Pennington, 1981).

Secondly, the basophil megakaryocyte phase, where the RNA synthesis becomes more marked. It is also clear from electron microscopic studies, that specific organelles are present in greater quantity and much more diffused throughout the cell than in the megakaryoblast phase (Pennington, 1981). By the end of this stage, endomitosis has ceased (Ebbe & Stohlman, 1965) and the mitochondria are of comparable size to those in platelets (Pennington & Streatfield, 1975).

Thirdly, the granular megakaryocyte phase in which the membrane system becomes open and communicates with the external environment (Behnke, 1968) and microtubule rich pseudopodia appear and penetrate the wall of the marrow sinusoid (Becker & de Bruyn, 1976; Lichtman et al., 1978).

The release of platelets from megakaryocytes into circulating blood may occur by either, or both, of two mechanisms The pseudopodia protruding through the sinusoidal wall may be removed by the circulating blood (Tavassoli, 1979), or alternatively, between 20% and 50% of the whole mature

megakaryocytes pass into the circulation (Kaufman et al., 1965). The site of transformation from megakaryocyte fraction to platelet is unknown, although it is believed that large megakaryocyte fragments trapped in small pulmonary vessels would be further fragmented releasing smaller fractions. These fractions are then classified as platelets when there is a regular arrangement of both microtubules and the dense tubular system, which corresponds to the characteristic discoid form of the platelet. This regular arrangement of intracellular organelles is absent from megakaryocyte fractions (Pennington et al., 1976). It has been estimated that about 3000 platelets are released from a single megakaryocyte.

Several radio-chemicals have been employed in vitro to assess the life-span of normal platelets. Studies using ^{51}Cr, believed to be the most reliable method, estimates an 8 to 11 day life time for the platelet (Harker & Slichter, 1972). The normal human blood platelet count is about 2.5×10^{8} cells per ml of whole blood and the turnover rate is estimated to be around 3.5×10^{7} cells/ml/day (Harker & Finch, 1969). Platelets are removed from circulating blood either by their incorporation into haemostatic plugs or by being engulfed by the liver or the reticulo-endothelial system in the spleen (Gordon, 1981).

3. Platelet morphology

The advent of electron microscopic techniques have allowed an insight into the complex nature of the intracellular environment of the platelet and have permitted the investigation of the relationship between the intracellular constituents and the physiological functions of the cell.

In their quiescent state the platelet exists in circulating blood as a biconvex disc with an equatorial diameter of 2-3 microns and a thickness of about 1 micron. The mammalian platelet has no nucleus and so differs from the analogous cell, the thrombocyte, in lower animals such as birds and reptiles.

The main features of the human platelet are diagramatically represented in Figure 1. The external surface of the plasma membrane (PM) is associated with the platelet surface coat or "glycocalyx" (GC) (Jamieson & Smith, 1976). This is a covering, 20-50 nm thick, rich in glycoproteins which appears fuzzy on electron micrographs when stained with lanthanum. The plasma membrane itself is a lipid structure of 7-10 nm thickness.

The external surface area of the platelet is markedly increased by the presence of the open canalicular system (OCS). These extensive and deeply penetrating invaginations give the platelet a "sponge-like" appearance and allow additional area for the actions of chemical and particulate stimuli (Gordon, 1981).

The major and most discernable of the cyto-skeletal proteins is the microtubular system (MT) which lies immediately beneath the plasma membrane in the equatorial plane. They appear to be polymeric assemblies of sub unit proteins of which the major one is tubulin (Crawford, 1985). The primary role of the microtubules seems to be to help maintain the discoid shape of the platelet. The contractile protein complex, thrombosthenin, isolated from platelets (Bettex-Galland & Luscher, 1959) was found to closely resemble the actomyosin of smooth muscle.

The fibrillar forms of actin, or microfilaments (MF), are seen both free in the cytoplasm and as a network of filaments attached to the inner plasma membrane. Myosin filaments, although present in the cytoplasm, are rarely observed (Crawford, 1985).

The dense tubular system (DTS) is situated distal to the marginal microtubular bundle. It is a system of membranes unconnected to the external milieu and probably analogous to the endoplasmic reticulum in other secretory, mammalian cells (Gerrard et al., 1981). The dense tubular system, like the endoplasmic reticulum, is possibly a Ca^{2+} sequestration organelle which serves as a source of intracellular Ca^{2+} which may be of importance in stimulus response coupling with particular importance in actomyosin contraction.

The platelet also contains multiple types of storage granules. Of these the three most distinct are the dense granules (DG), or dense bodies, the alpha granules (AG) and lysosomes (LYS). The term dense granule was coined due to their opaque appearance when viewed with an electron microscope (Tranzer et al., 1966). This is because these organelles are relatively Ca^{2+} rich, indeed it has been proposed that up to 60% of the cellular Ca^{2+} is associated with the dense granules (Skaer, 1975) although this may be a liberal estimation (Gerrard et al., 1981). This Ca^{2+} may be complexed in some way with the ADP which is present within the granule (Lages et al., 1975). The dense granule is also the storage site for 5HT within the cell. The platelet is a major source of 5HT within the body as it removes the amine from the circulation by an avid and specific active uptake process. In addition to

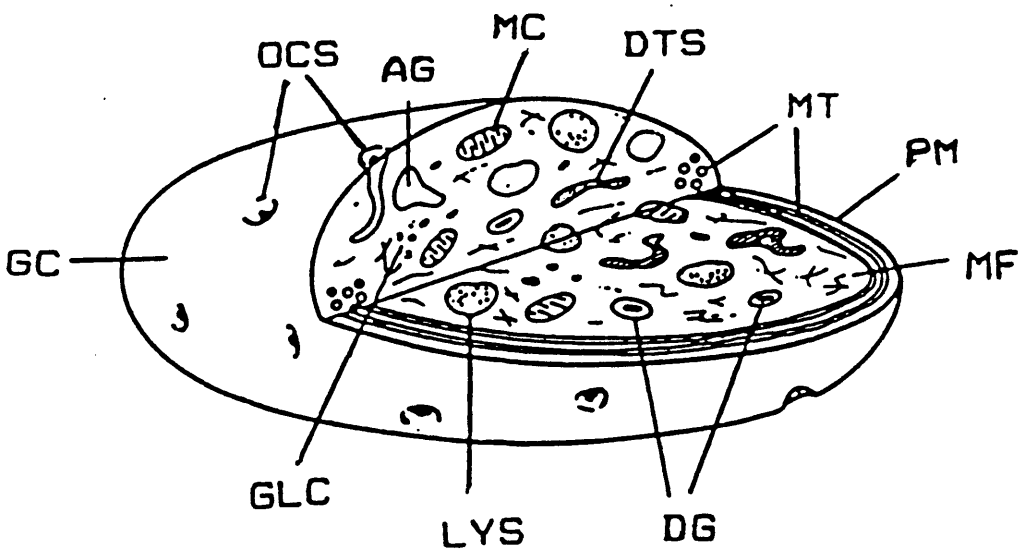

Figure 1. Diagramatic representation of the structure of the human platelet.

PM - Plasma membrane; GC - Glycocalyx; OCS - Open canalicular system;
MT - Microtubules; MF - Microfilaments; DTS - Dense tubular system;
DG - Danse granules; AG - Alpha granules; LYS - Lysosomes; MC - Mitochondria;
GLC - Glycogen granules. (After Crawford, 1985).

Ca^{2+}, 5HT and ADP the dense granules also contain ATP, pyrophosphate, phospholipid and antiplasmin. Due to its smallness relative to alpha granules and lysosomes, it is possible to quantify the dense granules within the platelet, a property relatively unique to its cell type. It has been estimated that there are 6-10 dense granules per platelet each with a diameter of around 198 nm (Costa et al., 1977).

The alpha granule appears to be the site where a variety of functionally significant proteins are localised. Included among these proteins are fibrinogen, fibronectin, thrombospondin (TSP), von Willebrand Factor, Platelet Factor 4, low affinity Platelet Factor 4, Factors V and Va, Factor VIII-related antigen, beta thromboglobulin, albumin, Platelet Derived Growth Factor (PDGF) and beta Transforming Growth Factor (BTGF) (Kaplan, 1981). Lysosomes contain a number of enzymes such as beta N-acetylglucosaminidase, beta galactosidase, beta glucuronidase and beta glycerophosphatase (Skaer, 1981).

The energy necessary for cellular reactivity derives from two major sources:-

a) Oxidative phosphorylation occurs in the mitochondria (MC) and this represents the major organelle for the maintenance of cellular ATP levels.

b) Glycolysis, which occurs in the cytosol, utilises glycogen, which is stored in granular form (GLC), as substrate.

4. Platelet reactivity

Damage to blood vessel walls with the resultant removal of, at least part of, the endothelial lining induces two processes

designed to terminate the loss of blood.

Firstly when platelets come into contact with the exposed collagen of the damaged vascular wall a number of phenomena occur which collectively constitute the platelet response and results in the formation of a primary haemostatic plug (Gordon, 1981). The platelet response can be divided into a number of separate but related processes: - i) shape change; ii) adhesion; iii) aggregation; iv) secretion. In addition to sub endothelial collagen, a number of soluble compounds and particulate fractions can, to varying degrees, stimulate the platelet either *in vivo* or *in vitro*.

The second procoagulant process is the release of tissue thromboplastin by the damaged vessel wall which initiates the activation of the coagulation cascade, leading to the formation of thrombin and the deposition of an insoluble fibrin network (Bevers *et al.*, 1985). This thrombin further activates platelets and the fibrin network enmeshes the platelet aggregate and converts the primary haemostatic plug into a stable thrombus.

4.1 Shape change

When platelets are challenged with agonists such as ADP, thrombin, 5HT or the calcium ionophore A23187, the initial response is a marked change from the distinct, quiescent discoid shape (Gordon & Milner, 1977). This rapidly occurring event involves the cell becoming orbicular with the formation of pseudopodia which emanate from the cell body. These pseudopodia may extend up to 10 microns from the cell and are

therefore larger than the platelet itself (Harris, 1981). As a consequence of this change there is a dramatic increase in the surface area of the platelet, although the cellular volume remains unaltered. This increase in surface area most probably results in a decrease in net charge density which presumably facilitates platelet to platelet interaction.

These extracellular events are accompanied by the re-organisation of the cytoskeletal elements within the cell. Each pseudopod contains a bundle of parallel microfilaments and also, occasionally, microtubules. In addition there is a contraction of the marginal microtubules resulting in the centralisation of the intracellular organelles, although they are still surrounded by a band of microtubules (Harris, 1981). Although these effects of the contractile proteins are believed to be Ca^{2+} dependent, the platelets do not require the presence of Ca^{2+} in the bathing medium to sustain shape change. This suggests that the Ca^{2+} requirement is fulfilled by the mobilisation of Ca^{2+} from intracellular storage granules, probably the dense tubular system.

4.2 Adhesion

The circulating platelet is indifferent to the intact vascular walls and to other blood constituents, such as erythrocytes and leucocytes. In contrast, exposure of sub-endothelial fibrillar collagen initiates activation of the platelet, which adheres avidly to the connective tissue (Gordon, 1981). This adhesion is a complex process which evidently depends upon the presence of the plasma protein von Willebrands

factor, or factor VIII. This factor, *per se*, has no effect on quiescent platelets, however the presence of denuded endothelium may modify its structure leading to its binding to, perhaps glycoprotein Ib on, the platelet membrane. In this way it has been postulated that von Willebrands factor forms a bridge between the subendothelium and the platelet (Berndt & Phillips, 1981). Platelets will also adhere to non-physiological surfaces, for example glass, and this is facilitated by the presence of fibrinogen.

4.3 Aggregation

Aggregation describes the formation of clumps of activated platelets on top of the initial adherent layer at the site of vascular damage. This is an interaction specific to platelets as it occurs to the virtual exclusion of other blood cell types. The development of these aggregates is not fully understood although the presence of a complex of glycoproteins IIb and IIIa on the platelet membrane appears to be mandatory as do the co-factors Ca^{2+} and fibrinogen. When quiescent platelets are challenged with agonists, notably ADP, there is an increase in fibrinogen binding sites on the plasma membrane and there is indirect evidence that the glycoprotein IIb/IIIa complex may participate in the function of these binding sites (Berndt & Phillips, 1981). This bound fibrinogen may then form an isthmus between platelets. Calcium is believed to be necessary to maintain fibrinogen binding, thereby helping to stabilise platelet to platelet interaction. Lectins and various clotting factors may also play a pro-aggregatory role in haemostasis.

Platelet aggregation can be designated as "primary", where the process is reversible, or "secondary", where the platelets form permanent clumps. The former is mediated entirely by the initiating agonist, whereas the latter although initiated by exogenous agents is propagated by endogenous mediators.

4.4 Secretion

Secretion from stimulated platelets can be broadly divided into two distinct categories. Firstly there is the selective release of storage granule contents, termed degranulation, exocytosis or merocrine secretion. The second mechanism involves the release of intracellular compounds which are not stored in their releasable form, but are synthesised and liberated upon activation (MacIntyre, 1977).

The platelet has the capacity to selectively release the constituents of one, or more, of its intracellular organelles indicating a fine level of control within the cell. The release of the dense granular contents occurs readily in response to relatively low concentrations of agonist, often in the absence of aggregation. In contrast the liberation of lysosomal enzymes is only induced by powerful stimuli such as thrombin. Release from alpha granules depends upon the concentration of the stimulus. Some of the alpha granular contents are liberated at lower agonist concentrations than are required for dense granule secretion, whereas others necessitate higher concentrations (Skaer, 1981).

It has been proposed that platelet stimulation causes dense granules to migrate to and fuse with the plasma membrane with the resultant liberation of the granule contents. In contrast the contents of the alpha granules are expelled into the open canalicular system. Furthermore it appears that alpha granule secretion depends not only on fusion with the open canalicular system but also with some form of platelet contraction. Little is known of lysosomal secretion, however some liberation of acid hydrolases occurs directly into internal phagocytic vacuoles (Lewis et al., 1976; Skaer, 1981).

The two principal examples of formation and release from stimulated platelets are eicosanoids and PAF. Platelets have an uptake process for, and are rich in, the eicosanoid precursor arachidonic acid (Rittenhouse-Simmons & Deykin, 1981). This unsaturated fatty acid is particularly prevalent at position 2 (sn-2) of the glycerol backbone of various phospholipids (Marcus et al., 1969). The arachidonic acid is released either by the action of phospholipase A_2, or by the sequential activities of phospholipase C and diacylglycerol lipase (Broekman et al., 1980; Bell & Majerus, 1980; MacIntyre, 1981). Regardless of the source of arachidonic acid it is susceptible to the action of two pathways, the cyclo-oxygenase and the lipoxygenase pathways. When platelets are challenged with potent stimuli, the cyclo-oxygenase endoperoxides prostaglandin (PG)G_2 and PGH_2, Thromboxane(Tx)A_2 and, latterly, the lipoxygenase products 12 L hydroxyperoxy- and 12 L hydroxy-5,8,10,14 eicosatetraenoic acid (HPETE/HETE) are the major eicosanoids formed and secreted (Rittenhouse-Simmons & Russell, 1978; Rittenhouse-Simmons & Deykin, 1981). Adrenaline and ADP are weak agonists in respect of their ability to induce eicosanoid

release, indeed the release in response to ADP is often undetectable (Best et al., 1980).

Similarly platelet stimulants such as thrombin, collagen and A23187 but not ADP induce the release of the ether lipid 1 O alkyl 2 acetyl sn glycero 3 phosphoryl choline (Platelet activating factor; PAF). The precise mechanism is unclear, although a phospholipase A_2 like enzyme may catalyse the deacylation of membrane phospholipid to synthesize lyso-PAF. Acetylation of this precursor at glycerol sn-2 would yield PAF (Chap et al., 1981; Chignard et al., 1983; Vargaftig et al., 1981; Chignard et al., 1985).

5. Platelets in vivo

5.1 Physiological functions

The primary function of the platelet is its involvement in the maintenance of vascular homeostasis (haemostasis). The initial response of the platelet, as outlined earlier, when encountering the exposed collagen of a damaged vascular wall is to adhere to the sub-endothelial connective tissue with the consequent formation of a primary haemostatic plug. In addition the platelet is involved in a variety of haemostatic mechanisms. The secretion of ADP, 5HT, TxA_2 and PAF, all of which are pro-aggregatory agents, act as a positive feedback stimulus to promote the activation of additional platelets with the consequent accumulation on the initial adherent layer. This is further enhanced by the release of PF4 and Ca^{2+}. Four major adhesive glycoproteins are released from the alpha granules of stimulated platelets - fibrinogen, fibronectin, von Willebrand Factor and thrombospondin (TSP). The TSP released rapidly binds to the platelet surface in the presence

of Ca^{2+} (Phillips et al., 1980) and is thought to reinforce the bridging fibrinogen molecule linking the GP IIb/IIIa complexes on adjacent platelets, leading to an irreversible state (Silverstein et al., 1986). The role of von Willebrand Factor, fibronectin and PF4, each of which are present on the surface of stimulated platelets, is unclear although a role in the stabilisation of aggregates is not precluded. This haemostatic plug is consolidated to form a thrombus by the initiation of the coagulation cascade, including the production of fibrin strands around the aggregate and the release of the powerful aggregatory agent thrombin (Gordon, 1981).

Following this rapid thrombus formation which serves to minimise blood loss, platelets are believed to become involved in the repair process. Proteins such as PDGF and BTGF are released which stimulate proliferation of smooth muscle, synthesis of connective tissue and formation of fibroblasts (Gordon & Milner, 1977). The 5HT secreted at the site of injury then stimulates these fibroblasts to release collagen (Aalto & Kulonen, 1972).

Platelets may also be involved in maintaining the integrity of the blood vessel endothelium. They achieve this by becoming integrated either at the junctions between endothelial cells or within the cells themselves (Johnson, 1971).

The possibility of platelets acting as scavengers in vivo has been raised as they are capable of pinocytosis and of the ingestion of several small particles. However it is unclear whether this is true phagocytosis or indeed if it is physiologically significant (Gordon & Milner, 1977; Skaer, 1981).

Platelets have been shown to accumulate at the site of tissue damage and it is believed that they play a role in the mediation of inflammation via both direct and indirect effects on vascular permeability. Directly the release of 5HT, PGD_2 and HETE all have a pronounced effect on vascular permeability. Indirectly the secretion from alpha granules may cause the degranulation of mast cells thereby releasing histamine which increases permeability (Gordon & Milner, 1977).

5.2 Pathological processes

The contribution of platelets to pathological conditions are an extension of their physiological functions.

Platelets play a major role in arterial thrombosis. This condition is due, at least in part, to an oversensitivity of platelets to pro-aggregatory agents. This results in the formation of thrombi either attached to vessel walls in the absence of vascular damage, or circulating without any vascular contact whatsoever. The consequence of this is that these thrombi, or fragments of them, block smaller vessels and so deprive tissues of oxygen. This is often fatal if the obstruction occurs in the myocardium or in the brain.

The condition of atherosclerosis is associated with cardiac, cerebral or peripheral ischaemia due to localised thickening of arterial intima. This thickening is accounted for by the formation of mural plaques which consist of fibrous tissue and lipid. Platelets may be involved in a variety of ways in the development of these plaques. Firstly, by forming

mural thrombi and being incorporated into the arterial wall; this may be particularly important in areas where there is disruption of the blood flow. Secondly, platelets may adhere to existing atherosclerotic plaques. Thirdly, by secretion of granular constituents which result in the proliferation of smooth muscle and formation of connective tissue; this is believed to be the major contribution that platelets play in atherosclerosis (Gordon & Milner, 1977; Born, 1985).

Platelets have also been implicated in conditions such as pathological inflammation, the rejection of organ transplants and migraine.

CHAPTER II: STIMULUS-RESPONSE COUPLING

1. General

The response of platelets to changes in their environment depends, in common with other cells, upon the interaction of external agents with their specific cell surface receptors. These agents, or agonists, can either inhibit platelet reactivity thereby maintaining or prolonging the quiescent state, or stimulate platelet reactivity, and hence initiate activation, of the platelet. The information is transferred from the external stimulus, which can be soluble or particulate, to the final cellular response by a process known as stimulus-response coupling. The general mechanism involved is shown in Figure 2. The agonists bind to their specific receptors which are situated on the external surface of the plasma membrane. Information generated as a consequence of agonist receptor combination is transmitted through the membrane by a trasduction process. These transducers are believed to be a family of heterotrimer proteins which are inert until they bind guanosine triphosphate (GTP) and are therefore termed "G-Proteins" (Rodbell, 1980). When GTP binds to the G-proteins the complex activates an effector, normally an enzyme, on the inner face of the membrane. This enzyme amplifies the signal by converting an intracellular precursor into an active chemical, or second messenger, which may diffuse into the cytoplasm or remain within the plasma membrane. The role of this second messenger is to stimulate specific intracellular target sites, or acceptors, which in turn are responsible for the final cellular response. There appears to be only a limited number of naturally occurring second messengers, suggesting that internal transduction

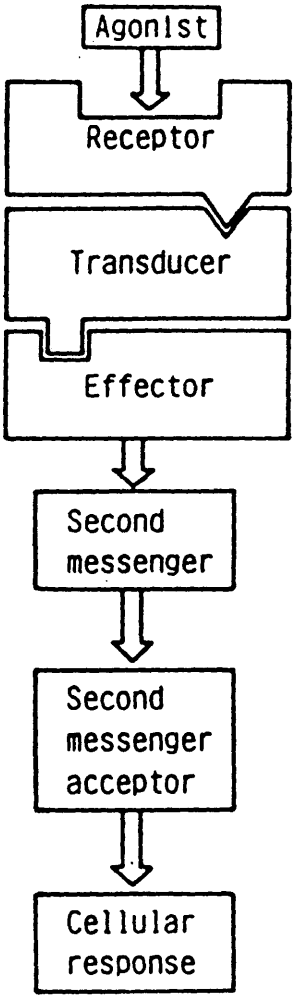

Figure 2. General mechanism of stimulus response coupling. See text for details.

processes are relatively universal (Berridge, 1985). As a result of this, the formation of the same second messenger(s) may be the common product of a variety of agonists and therefore a similar cellular response can be observed from a plethora of external stimulants. The most important second messengers so far discovered are cAMP, 1,2 Diacylglycerol (DAG), inositol 1,4,5 trisphosphate ($Ins(1,4,5)P_3$) and Ca^{2+} (Schramm & Selinger, 1984; Berridge, 1984). As mentioned above, platelet agonists may be either stimulatory or inhibitory. In consequence the corresponding second messenger systems also can be designated either inhibitory or stimulatory (see Table 1).

2. Inhibition

Platelet inhibition, that is the prevention of function, is associated with the activation of adenylate cyclase and consequent elevation of the intracellular concentration of cAMP. Each of the inhibitory agonists in Table 1 have been shown to stimulate cAMP formation (Feinstein *et al.*, 1981; Aktories & Jakobs, 1985).

2.1 Agonists and receptors

2.1(a) Prostacyclin:

Prostacyclin (PGI_2) is synthesised by and secreted from, vascular endothelial cells (Moncada & Vane, 1979) and is the most potent endogenous anti-aggregatory agent known. It is formed by the action of PGI_2 synthetase on the PG endoperoxides, however it is highly unstable and this has restricted the anti-thrombotic potential of exogenous PGI_2 *in vivo*. Binding

AGONIST	RECEPTOR	FUNCTION
Prostacyclin	IP	Inhibit
Prostaglandin E_1	IP	Inhibit
Prostaglandin D_2	DP	Inhibit
Adenosine	A_2	Inhibit
Adrenaline	β_2	Inhibit
Adrenaline	α_2	Stimulate
Thrombin	??	Stimulate
ADP	P_{2T}	Stimulate
PAF	P_{af_1}	Stimulate
Vasopressin	V_1	Stimulate
Thromboxane A_2	TP	Stimulate
5HT	$5HT_2$	Stimulate

Table 1. Platelet inhibitory and stimulatory agonists.

A selection of those agonists which either stimulate or inhibit human platelet functional responses, together with their receptor sub-types.

studies have revealed two distinct PGI_2 sites on the human platelet. The first is a high affinity, low capacity (50 sites/cell, Kd = 10 nM) site which possesses the pharmacological properties of the functional receptor and the second is a low affinity, high capacity (4000 sites, Kd = 1.6 μM) site (Siegl, 1982; Whittle et al., 1985). The PGI_2 receptor conforms to the IP class of prostanoid receptor (Kennedy et al., 1983).

2.1(b) Prostaglandin E_1:

Prostaglandin (PG) E_1 is an inhibitor of platelet function some 30 times less potent than PGI_2. Pharmacological studies in intact platelets and in lysates have indicated that PGE_1 is an agonist at the IP receptor. Pre-exposure of the cells to PGI_2 render them insensitive to a subsequent addition of either PGI_2 or PGE_1 and in addition both eicosanoids displace $[^3H]$-PGE_1 from the receptor at comparable concentrations (Whittle et al., 1985; MacIntyre et al., 1986).

2.1(c) Prostaglandin D_2:

PGD_2 is an anti-aggregatory, vasoconstrictory and anti-neoplastic agent. It is 10 times less potent than PGI_2 in respect of anti-aggregatory activity. Radioligand analyses have identified a single class of binding site (760 sites/cell). Structure activity relationships suggest that this site, designated DP, differs from those mediating vasoconstriction and anti-neoplastic activities (Whittle et al., 1985; MacIntyre et al., 1986).

2.1(d) Adenosine:

Adenosine at low concentrations inhibit platelet function. The presence of an adenosine uptake process in the platelet has until recently hindered binding studies. However, studies utilising a novel adenosine analogue indicates that the receptor conforms to the A_2 class of adenosine receptors (Ukena et al., 1986). In addition to this extracellular receptor there may be an intracellular receptor, probably a P_1 purino-receptor, which may be activated at higher concentrations and oppose the actions of the extracellular receptor (MacIntyre et al., 1986).

2.1(e) Adrenaline:

As indicated on Table 1, adrenaline can either activate or inhibit the platelet depending on which receptor is activated. The α_2 mediated activation is the dominant response in the human platelet, however the β specific agonist isoprenaline inhibits responses to agonists such as ADP. The use of ligand binding studies, selective agonists and antagonists indicate that the receptor is of the β_2 subtype and that there are 25-66 receptors per platelet (Kerry & Scrutton, 1985).

2.2 G proteins

The inhibitory compounds, PGI_2, PGE_1, PGD_2, adenosine and β_2 specific agonists all exert their activity via the stimulation of adenylate cyclase and the consequent production of cAMP (Aktories & Jakobs, 1985). The transduction process mediating adenylate cyclase activity involves a pair of G proteins (see Figure 3). The first, termed N_s, is associated with receptors which stimulate adenylate cyclase. In contrast

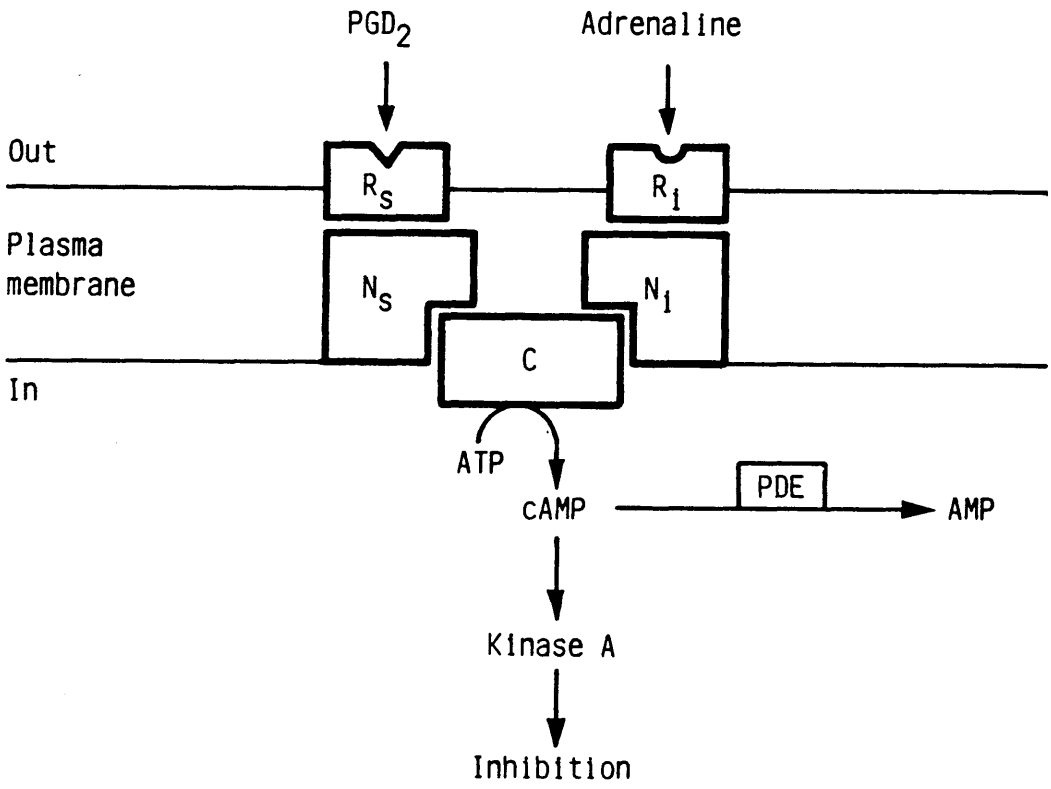

Figure 3. Regulation of cyclic AMP formation by platelets.
See text for details.

N_i is responsible for agonist induced inhibition of the enzyme (Rodbell, 1980). Each of these G proteins consist of three subunits alpha, beta and gamma and in both cases beta and gamma are identical.

The binding of an inhibitory agonist to its receptor induces a conformational change in the receptor. This change is transmitted through to N_s which becomes susceptible to GTP which approaches from within the cell. The interaction of GTP constitutes the "on reaction" enabling it to activate adenylate cyclase (Berridge, 1985). The precise mechanism is unclear although it is believed that some form of inhibition of the active α subunit is exerted by the beta-gamma complex and this inhibition is removed by receptor occupancy (Jacobs et al., 1986). The activity of the active species of N_s is terminated by the hydrolysis of the GTP to GDP by a GTPase inherent in the α subunit.

A similar series of events occurs during the inhibition of adenylate cyclase by N_i. The effect of an inhibition of adenylate cyclase would be to decrease the cAMP levels within the cell, although the functional significance of this in the platelet is controversial.

The alpha subunits are targets for the actions of cholera and pertussis toxins. Cholera toxin catalyses the ADP-ribosylation of the alpha subunit of N_s. This has the effect of inhibiting the GTPase activity of N_s, therefore there is no hydrolysis of GTP and so N_s is permanently active increasing the cellular cAMP content (Cassel & Selinger, 1977). Similarly

pertussis toxin ADP-ribosylates N_i, prevents its activation and so prevents those agonists which would inhibit cellular activity having any effect (Jakobs et al., 1986). In fact platelets do not contain the appropriate receptor (ganglioside) to which the toxins must bind before being internalised and therefore neither can be studied in intact platelets, however they have been studied in platelet membrane fractions.

2.3 Adenylate cyclase

The effector mediating inhibition in the platelet is adenylate cyclase. This is the enzyme (mol.wgt. 150,000) which converts a metal-ATP complex, most probably MgATP, into the intracellular second messenger cAMP. It appears to be a glycoprotein which traverses the plasma membrane, although the physiological significance of this is unknown (Pfeuffer et al., 1985). The enzyme can be directly activated by forskolin, a diterpine isolated from the indian plant Coleus forskohlii, which elevates cAMP in intact platelets and inhibits platelet aggregation (Insel et al., 1982).

2.4 Cyclic AMP

The product of stimulated adenylate cyclase activity is elevated cytoplasmic levels of cAMP. When synthesised this cyclic nucleotide diffuses into the cytoplasm and activates a cAMP dependent protein kinase(s) (Krebs & Beavo, 1979). There is evidence however that it is not the total cellular cAMP content which is critical for the activity of these kinases but the activity of a smaller specific cAMP pool. The specific

targets for these kinases have not been delineated, however the phosphorylation (and any subsequent dephosphorylations) are not regarded as simple "on-off" mechanisms. The alterations in the levels of protein phosphorylation regulated by the kinases serves as a precise method of regulating cellular response (Cohen, 1982). The cAMP is metabolised to 5'AMP by the action of a cAMP-specific phosphodiesterase. Non specific phosphodiesterase inhibitors, such as isobutyl methyl xanthine (IBMX), prevent cAMP metabolism and potentiate the inhibitory effects of adenylate cyclase stimulants.

2.5 Site of inhibition

Acting through cAMP dependent protein kinase activities, cAMP potentially may mediate inhibition of the platelet activation pathway in a variety of ways. The precise site of cAMP dependent regulation is unknown but it could be at one or more of the following points:-

a) It may interfere with the platelet stimulatory transduction process thereby limiting the production of various second messengers. It has been shown to interfere with phospholipase C activity (Lapetina et al., 1977) and the consequent production of inositol phosphates (Watson et al., 1984).

b) It may enhance the removal, or metabolism of stimulatory second messengers and so terminate their effects. Cyclic AMP inhibits and reverses agonist induced elevation of cytosolic Ca^{2+} levels (Feinstein et al., 1983; Zavoico & Feinstein, 1984; MacIntyre et al., 1985a). In addition cAMP stimulates the active sequestration of Ca^{2+} into purified platelet intracellular

membrane preparations (Kaser-Glanzmann et al., 1977).

c) It may impede protein phosphorylation (or stimulate dephosphorylation) or indeed inhibit the activity of those phosphorylated proteins required for cellular reactivity. In support of this PGE_1 inhibits the phosphorylation of proteins which have been implicated in platelet activation (Lyons et al., 1975).

3. Stimulation

The obvious converse to the observation that elevated cAMP levels inhibit platelet function, is that a decrease in the resting cAMP levels activate the platelet (Salzman, 1972). However, no agonist has been shown to have such an effect, although adrenaline and ADP both reverse cAMP levels elevated by adenylate cyclase stimulants (Mills, 1974). In addition thrombin, adrenaline, ADP, vasopressin and TxA_2 all inhibit adenylate cyclase in platelet membranes (Aktories & Jakobs, 1985; Vanderwel et al., 1983; Miller & Gorman, 1976). The functional significance of stimulatory agonists on total cAMP content is unclear although the possibility that adenylate cyclase inhibition is involved in activation must be taken into account (Aktories & Jakobs, 1985). Since there are distinct compartments of cAMP in platelets (Hashimoto, 1983), the possibility exists that regulation of adenylate cyclase by agonists may play an important role in cellular reactions in the absence of demonstrable changes in the overall level of cAMP.

Stimulation of human platelets, in common with other secretory cells, has been associated with a rise in the levels of cytoplasmic calcium ($[Ca^{2+}]_i$) (Gerrard et al., 1981). In 1975, Michell related increased $[Ca^{2+}]_i$ to the hydrolysis of the phospholipid phosphatidylinositol (Ptd Ins) and hypothesised that this phospholipase C catalysed event serves as a transduction process leading to the gating of Ca^{2+} (Michell, 1975). It is now accepted that the agonist stimulated hydrolysis of a family of inositol phospholipids generates two products (DAG and Ins(1,4,5)P_3) both of which may function as second messengers in stimulated cells. Ins(1,4,5)P_3 seems to act by mobilizing intracellular Ca^{2+} stores, whereas DAG stimulates protein phosphorylation (Berridge, 1984). Platelet stimulatory agonists such as thrombin, PAF, 5HT, TxA_2 and vasopressin are believed to exert their effects at least in part by such a mechanism.

3.1 Agonists and receptors

3.1(a) ADP:

ADP is a platelet stimulatory agonist which induces the whole range of functional responses. Binding studies, using stable analogues due to the instability of the parent compound, indicate a single class of binding site with 400-1200 sites per cell (Macfarlane et al., 1983). It appears that the platelet ADP receptor is unique and has been designated P_{2T} (Gordon, 1986).

3.1(b) 5HT:

Platelets remove 5HT from the blood by an avid and specific uptake system. In addition this biogenic amine is a weak platelet stimulant acting on specific receptors (probably 260 per cell) which are of the $5HT_2$ subtype (de Clerk et al., 1984; de Chaffoy de Courcelles et al., 1985).

3.1(c) Thromboxane A_2:

TxA_2 is synthesised in and released from stimulated platelets (Rittenhouse-Simmons & Deykin, 1981) and acts as a pro-aggregatory and vasoconstrictor agent. The effects of TxA_2 on platelets are shared by the prostaglandin endoperoxides PGG_2 and PGH_2. The instability of these compounds led to the synthesis of a battery of stable analogues such as U44069, U46619 (Bundy, 1975) and EP171 (Jones et al., 1985). Although two binding sites exist on platelet plasma membranes, only one (2000 sites per platelet) relates to the pharmacological characteristics of receptor mediated activation (MacIntyre et al., 1986). This receptor is of the TP class of prostanoid receptor (Kennedy et al., 1983) and is linked to inositol phospholipid hydrolysis.

3.1(d) Platelet activating factor:

The biologically active ether lipid, PAF, has dramatic cardiovascular and bronchopulmonary effects in experimental animals, including hypotension and bronchoconstriction. At the cellular level PAF stimulates, amongst others, rat, rabbit and human neutrophils and also rabbit and human platelets

(Roth, 1986). Numerous studies have failed to conclude whether there are multiple types of PAF binding sites on the platelet plasma membrane. However on the basis of the antagonistic effect of kadsurenone, the PAF receptor on the human platelet (termed Paf_1) differs from that on the macrophage (termed Paf_2) (Lambrecht & Parnham, 1986).

3.1(e) Vasopressin:

Vasopressin is the most potent endogenous pressor agent known and possesses anti diuresis activity. In addition vasopressin stimulates the human platelet via a specific receptor although the physiological significance of this activation is unclear. There are around 90-150 sites per platelet (Pletscher et al., 1985) and, in common with vascular smooth muscle, they are the inositol phospholipid linked V_1 type (Siess et al., 1986; Pollock & MacIntyre, 1986).

3.1(f) Thrombin:

Thrombin is the most efficacious and potent platelet agonist. It is a serine protease with catalytic activity against the carboxyl linkage of arginine residues and this activity is a pre-requisite for platelet activation. Two classes of binding site have been identified on the platelet, a high affinity (500 sites, Kd = 10 nM) and a low affinity (50,000 sites, 100 nM). It has been proposed that one of these sites is Glycoprotein V which is the only known thrombin substrate on the plasma membrane. Although the high affinity site correlates in many respects to platelet activation, the functional significance of the binding sites has been challenged. It

is possible to remove almost all of the Glycoprotein V without impairing thrombin induced platelet activation. Inactive thrombin, that is with the active site blocked, as well as active thrombin binds to the platelet and thrombin analogues can stimulate without detectable binding. This suggests that active thrombin modulates the receptor and stimulates the platelet without remaining bound to the receptor (Detwiler & McGowan, 1985). The discovery of a partial agonist which inhibits thrombin induced activation with no effect on its proteolytic activity (Ruda et al., 1985), perhaps indicates a twin stimulation via both an agonist receptor interaction and a catalytic activity (MacIntyre et al., 1986).

3.1(g) Adrenaline:

The stimulatory effects of adrenaline are mediated by the α_2 adrenoceptor of which there are around 270 sites per platelet (Kerry & Scrutton, 1985). Adrenaline causes both platelet aggregation itself (O'Brien, 1963) and potentiates aggregation induced by other agents (Mills & Roberts, 1967). However adrenaline induced aggregation differs from other agonists as it is not preceded by shape change. In addition, adrenaline induced activity is not associated with an increase in $\left[Ca^{2+}\right]_i$ (MacIntyre et al., 1985b) and the α_2 receptor is linked, via N_i, to inhibition of adenylate cyclase (Aktories & Jakobs, 1985).

3.2 G proteins

The involvement of G proteins as the transduction process in the inositol phospholipid mediated activation of platelets has not been conclusively demonstrated. In platelets permeabilise by high voltage electric charges, Ca^{2+} induces 5HT release and this release is greatly potentiated by GTP and its analogues (Haslam & Davidson, 1984a). In addition, in these platelets there is increased production of DAG in the presence of GTP (Haslam & Davidson, 1984b). This suggests that G proteins do play a role in platelet stimulation. It is unclear whether these G proteins are identical to, or interact with, those involved in the regulation of the adenylate cyclase system.

3.3 Calcium

Ringer, in 1883, demonstrated the absolute dependence on Ca^{2+} for continued contraction of the heart. In further classical studies Katz (Katz, 1969) and Douglas (Douglas, 1974), amongst others, observed that physiological responses required extracellular Ca^{2+} and this prompted the proposal that many agonist evoked responses involved Ca^{2+} as a second messenger.

Calcium is perhaps the most extensively investigated and generally accepted intracellular second messenger in platelets (Gerrard *et al.*, 1981). The evidence for a central role for Ca^{2+} in platelets is partly circumstantial (by analogy with other cells) and partly indirect. Calcium is clearly a primary coupling agent in contractile tissue and secretory cells (Rasmussen & Barrett, 1984) and therefore by analogy a role has been postulated for it in platelets. Indirect evidence

for the involvement of Ca^{2+} stems from the ability of the ionophore A23187 to activate platelets in a manner similar to thrombin (Feinman & Detwiler, 1974; White et al., 1974). The mechanism of action of A23187 is not clear and is complicated as A23187 activates platelets equally well in an extracellular medium containing 1 mM Ca^{2+} or 1 mM EGTA, a Ca^{2+} chelating agent which renders the bathing medium nominally Ca^{2+} free (Feinman & Detwiler, 1974). Further evidence for a role of Ca^{2+} in platelet activation is provided by the demonstration that a variety of drugs which have been proposed to block calmodulin mediated, calcium dependent processes also block platelet responses to several agonists (Huang & Detwiler, 1986a).

The advent of the fluorescent calcium indicator dye, Quin 2 (Tsien et al., 1982) allowed, for the first time, the quantification of both basal and stimulated $[Ca^{2+}]_i$. Rink and colleagues concluded that in quiescent platelets the resting $[Ca^{2+}]_i$ was about 100 nM. When challenged with thrombin or ionomycin this rose to 3 μM, in the presence of 1 mM extracellular Ca^{2+}, or to 200-300 nM in its absence (Rink et al., 1982). This may suggest that agonists stimulate both the influx of Ca^{2+} from the external environment and mobilise Ca^{2+} from internal stores (MacIntyre et al., 1985b). Although dense granules, mitochondria, alpha granules and the internal surface of the plasma membrane all contain Ca^{2+} (Murer, 1969; Sato et al., 1975), it is popularly believed that the dense tubular system is the source of this mobilised Ca^{2+} (White, 1972). Shape change, dense granular secretion and aggregation

induced by ionophore was observed to occur at $[Ca^{2+}]_i$ levels of 0.5, 0.8 and 2 μM respectively. However this correlation was not absolute as thrombin stimulated more secretion than an ionophore concentration which elicited a comparable rise in $[Ca^{2+}]_i$. In addition thrombin induced shape change of platelets at $[Ca^{2+}]_i$ values below the threshold (0.5 μM) described above. This indicates that agonists must evoke additional mechanisms or second messengers besides the evoked increase in $[Ca^{2+}]_i$.

The mechanism(s) underlying the agonist induced increase in $[Ca^{2+}]_i$ have been the subject of numerous studies. The translocation of Ca^{2+} from the extracellular space to the intracellular environment is believed to be achieved by the opening of specific calcium channels in the plasma membrane allowing the Ca^{2+} to enter the cell down its concentration gradient. There are a variety of such channels in biological tissues. There are voltage operated channels (VOCs) which are opened by the depolarisation of the cellular membrane. These VOCs are particularly prevalent in tissues such as smooth muscle (Bolton, 1979). The second type of channel are termed receptor operated (ROCs) and are opened as a direct consequence of receptor occupancy. In the case of human platelets, receptor mediated activation occurs in the absence of any detectable change in the membrane potential. Consequently agonist induced Ca^{2+} influx must occur via ROCs (MacIntyre & Rink, 1982; Doyle & Ruegg, 1985). The mobilisation of internal Ca^{2+} stores must depend upon the generation of

intracellular mediators which cause the liberation of the Ca^{2+} from the bound pool. Studies concerned with elucidating the mechanism of both Ca^{2+} influx and mobilisation have centered on the metabolism of a family of acidic phospholipids, the phosphoinositides.

3.4 The phosphoinositide cycle

The original concept expounded by Michell, was that phospholipase C activity resulted in the hydrolysis of Ptd Ins to form DAG and a mixture of two water soluble products inositol 1 phosphate (Ins 1P) and inositol 1,2-(cyclic) phosphate (Ins(1,2-cyc)P). DAG would be rapidly phosphorylated, by DAG kinase, to phosphatidic acid (Ptd OH) which, in turn, is converted to CDP-DAG by CTP (Ptd OH) cytidyl transferase. This CDP-DAG would combine with free inositol, liberated from the inositol phosphates by phosphatases, to reform Ptd Ins, under the action of CDP-DAG inositol transferase, thereby completing what was popularly termed the "Ptd Ins cycle". Michell proposed that receptor occupancy accelerated the rate of turnover of this cycle and by some mechanism, possibly due to ionophoric actions of Ptd OH (or of lyso Ptd OH), this would increase the permeability of the cellular membrane to the Ca^{2+} which mediated the final response (normally secretion). The intervening eleven years has shown that, not only is this system present in cells as diverse as brain (Berridge et al., 1983) and insect salivary gland (Berridge, 1983), but also that the process is a great deal more complex than outlined above. Figure 4 details

what has been shown, and what has been proposed, to occur in a broad spectra of cells including platelets.

Ptd Ins and its phosphorylated derivatives Ptd Ins 4 phosphate (PIP) and Ptd Ins 4,5 bisphosphate (PIP_2) (Figure 5) are relatively minor constituents of the mammalian plasma membrane representing about 5% of the total cellular phospholipid (Marcus et al., 1969). Ptd Ins is a typical phospholipid, situated primarily on the inner leaflet of the membrane bilayer. However it is unusual as it forms two derivatives by a pair of apparently futile, rapid turnover cycles. Ptd Ins kinase phosphorylates Ptd Ins, at position 4 on the sugar ring, to yield PIP which is further phosphorylated at position 5 to form PIP_2. Conversely these phosphate groups are removed by PIP phosphatase and PIP_2 phosphatase respectively. Ptd Ins remains the major inositol phospholipid representing 90% of the total phosphoinositide pool. The polyphosphoinositides, especially PIP_2, have the unique physicochemical property of having a very high affinity for Ca^{2+} (greater than that of EDTA) (Hirasawa & Nishizuka, 1985).

It has been shown in a variety of cells that the first inositol phosphate to be detected after agonist addition is Ins(1,4,5)P_3 (Figure 6) and not Ins 1P (Berridge, 1983; Martin, 1983; Drummond et al., 1984). In addition when platelets were challenged with thrombin this initial accumulation of Ins(1,4,5)P_3 coincides with decreased levels of PIP_2 (Agranoff et al., 1983). As a consequence of such studies it is now believed that it is PIP_2 which is initially hydrolysed by phospholipase C in response to agonist receptor interaction.

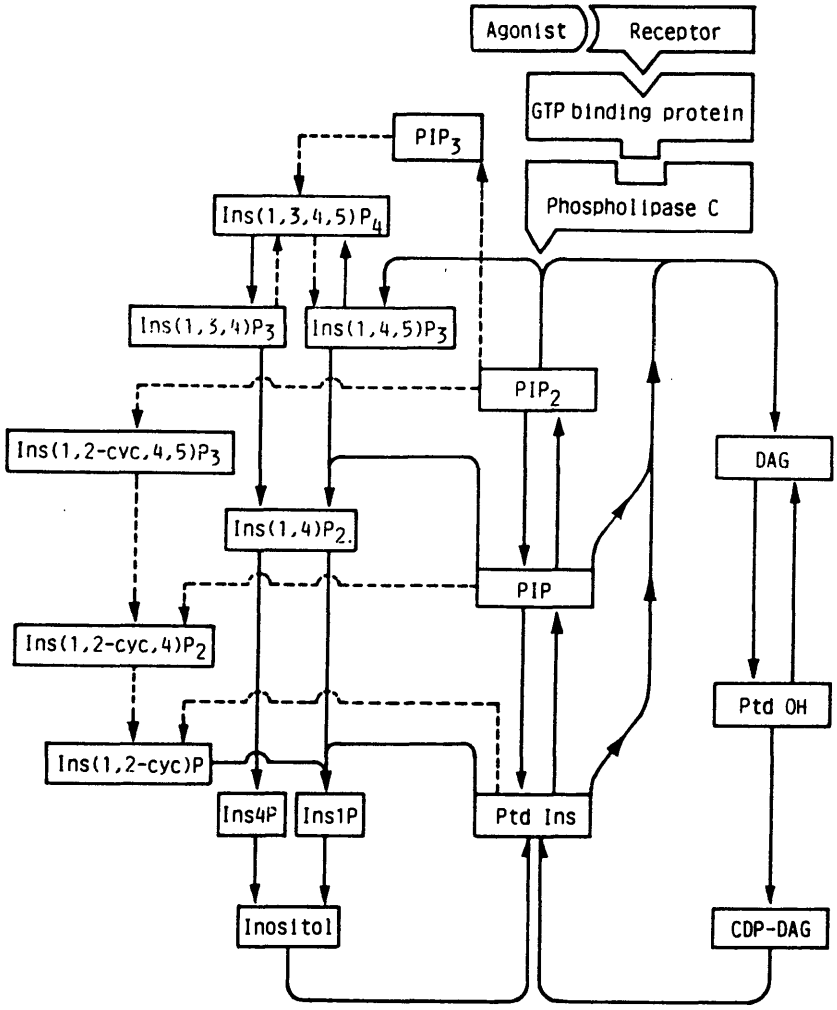

Figure 4. The phosphoinositide cycle.

This representation demonstrates the complex nature of the cycle. Receptor mediated phospholipase C activity results in the hydrolysis of one or more of the inositol phospholipids with the generation of DAG and, possibly a variety of inositol phosphates. The inositol phosphates and DAG are then incorporated in cycles leading to the resynthesis of the phosphoinositides.

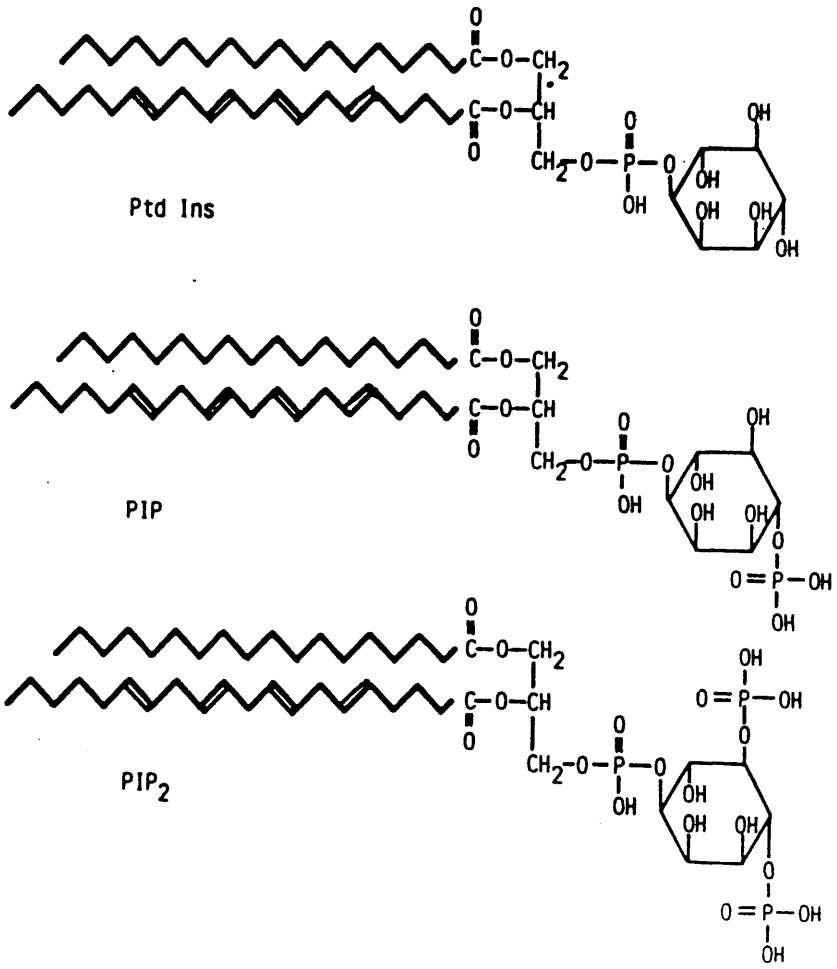

Figure 5. Structures of the inositol phospholipids.

Ptd Ins-Phosphatidylinositol; PIP-Phosphatidylinositol 4 phosphate; PIP_2-Phosphatidylinositol 4,5 bisphosphate.

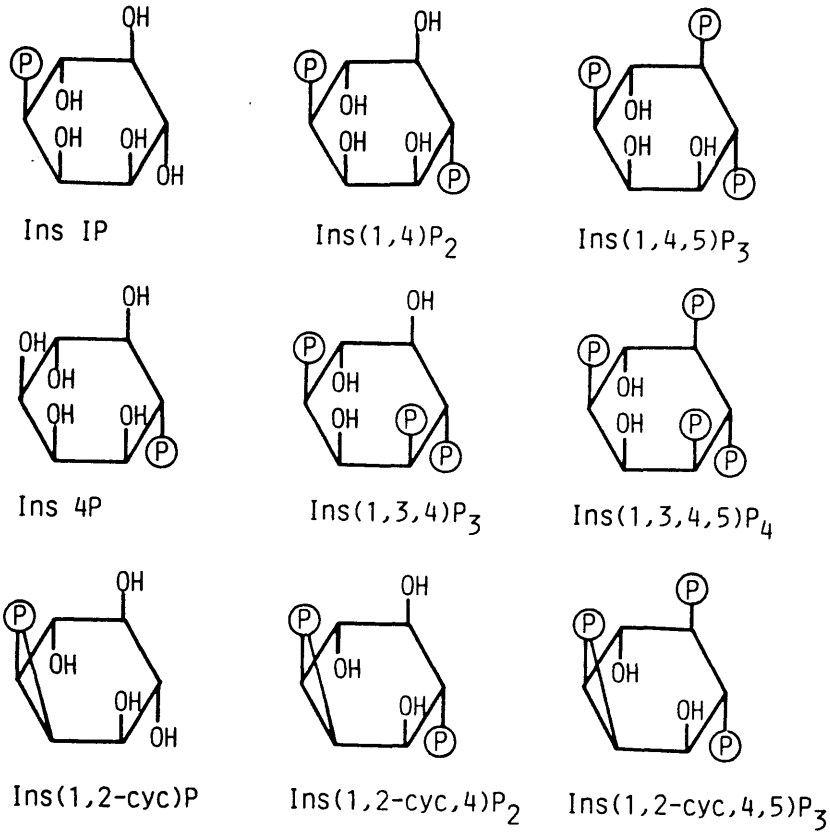

Figure 6. Structure of inositol phosphates.

InslP-Inositol 1 phosphate; Ins(1,4)P_2-Inositol 4,5 bisphosphate; Ins(1,4,5)P_3-Inositol 1,4,5 trisphosphate; Ins 4P-Inositol 4 phosphate; Ins(1,3,4)P_3-Inositol 1,3,4 trisphosphate; Ins(1,3,4,5)P_4-Inositol 1,3,4,5 tetrakisphosphate; Ins(1,2-cyc)P-Inositol 1,2-(cyclic) phosphate; Ins(1,2-cyc,4)P_2-Inositol 1,2-(cyclic) 4 bisphosphate; Ins(1,2-cyc, 4,5)P_3-Inositol 1,2-(cyclic) 4,5 trisphosphate.

The role of Ptd Ins and of PIP in platelet activation is the subject of some controversy. There is a marked decrease in the levels of both phospholipids in response to agonist stimulation although this occurs significantly later than that of PIP_2. In addition the formation of $Ins(1,4,5)P_3$ and inositol (1,4) bisphosphate ($Ins(1,4)P_2$) is followed by increased levels of Ins 1P and free inositol (Agranoff et al., 1983; Watson et al., 1984; Siess & Binder, 1985). These observations are consistent with one of two possible mechanisms. There may be direct hydrolysis of Ptd Ins and PIP, which would liberate Ins 1P and $Ins(1,4)P_2$ respectively, occurring after the initial PIP_2 breakdown (Wilson et al., 1985c; Majerus et al., 1985). Alternatively Ptd Ins may act as a phospholipid pool which replenishes the PIP_2 hydrolysed (Michell et al., 1981). Thus the lower phosphorylated inositol derivatives would be formed by dephosphorylation of $Ins(1,4,5)P_3$ (Downes et al., 1982; Berridge et al., 1983).

The enzymes which convert $Ins(1,4)P_2$ to Ins 1P and Ins 1P to myo-inositol are believed to be inhibited by lithium (Hallicher & Sherman, 1980; Huang & Detwiler, 1986b) and this blockade would limit the supply of inositol for phospholipid resynthesis. However a second metabolic pathway might exist which involves the conversion of $Ins(1,4)P_2$ to inositol 4 phosphate (Ins4P) and inositol (Storey et al., 1984) and this pathway is believed to be insensitive to lithium although the relative importance of each is unknown (Michell, 1986).

The complexity of the system is further indicated by reports of the accumulation of the inositol (1,3,4) trisphosphate ($Ins(1,3,4)P_3$; Figure 6) isomer in stimulated cells (Irvine et al., 1985; Burgess et al., 1985) although this has not, as yet, been demonstrated in platelets. The formation of $Ins(1,3,4)P_3$ occurs after that of $Ins(1,4,5)P_3$ as does its decline on removal of the stimulus. The source of $Ins(1,3,4)P_3$ is now thought to be inositol 1,3,4,5 tetrakisphosphate ($Ins(1,3,4,5)P_4$; Figure 6). $Ins(1,3,4,5)P_4$ is formed as rapidly as $Ins(1,4,5)P_3$ in rat cerebral cortical slices challenged with muscarinic agonists (Batty et al., 1985). There is evidence that $Ins(1,3,4,5)P_4$ is formed from $Ins(1,4,5)P_3$ by the activity of an ATP dependent kinase (Michell, 1986). Hence the metabolism of $Ins(1,4,5)P_3$ via $Ins(1,3,4,5)P_4$ and $Ins(1,3,4)P_3$ may be quantitatively as important as its dephosphorylation to $Ins(1,4)P_2$ (Michell, 1986). The alternative is that another, as yet undetected, inositol phospholipid (PIP_3) may exist, the hydrolysis of which would directly yield $Ins(1,3,4,5)P_4$ (Batty et al., 1985).

Until recently the only cyclic phosphate believed to be synthesised was Ins(1,2-cyc)P. However in studies using less acidic extraction procedures, Majerus and colleagues demonstrated the isolation and characterisation of inositol 1,2-(cyclic) 4-bisphosphate ($Ins(1,2\text{-cyc},4)P_2$) and inositol 1,2-(cyclic) 4,5-trisphosphate ($Ins(1,2\text{-cyc},4,5)P_3$), the two cyclic phosphate products of PIP and PIP_2 respectively (Wilson et al., 1985a,b). Indeed it has been proposed that $Ins(1,2\text{-cyc},4,5)P_3$

is the major product of phosphoinositide metabolism in thrombin stimulated platelets (Majerus et al., 1986).

In contrast to the situation involving the formation of inositol phosphates (Figure 6), the formation of DAG, Ptd OH and CDP-DAG (Figure 7) remains identical to Michell's original proposal. The only point of contention is the source of DAG as it can be formed by the action of phospholipase C on one or all of the inositol phospholipids. DAG is converted by DAG kinase to Ptd OH utilising ATP. Ptd OH is, along with CTP, then converted to CDP-DAG by CTP (Ptd OH) cytidyl transferase which combines with myo-inositol (from whatever source) to reform Ptd Ins.

3.5 Phosphatidic acid and lyso phosphatidic acid

Initially the prime candidate for an intracellular mediator of elevated $[Ca^{2+}]_i$ was Ptd OH. Ptd OH was shown to be a Ca^{2+} ionophore (Tyson et al., 1976) and therefore it was proposed that it was important in Ca^{2+} mediated cellular activation (Michell et al., 1977). There are however several lines of evidence which would challenge this as being the sole mechanism involved in the elevation of $[Ca^{2+}]_i$. Firstly, the formation of Ptd OH may not occur rapidly enough to account for the Ca^{2+} influx and secondly it is now debatable whether Ptd OH does possess ionophoric activity (Holmes & Yoss, 1983) or increase Ca^{2+} permeability (Putney, 1986).

Lyso Ptd OH, which is formed in the platelet by the activity of phospholipase A_2 on Ptd OH, is a potent pro-

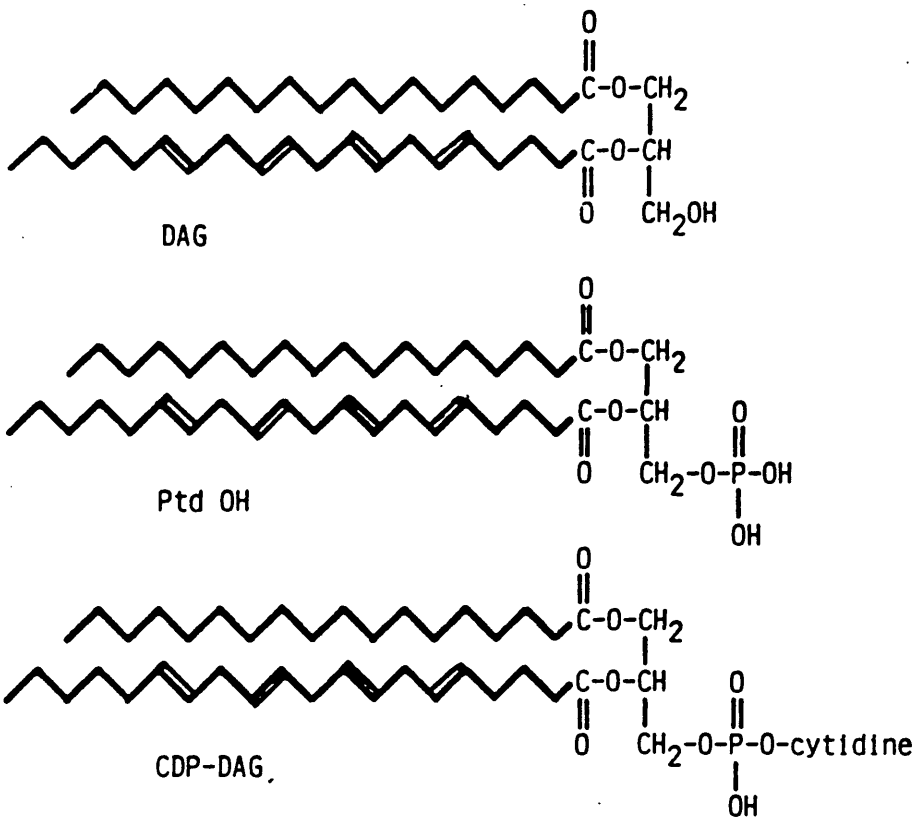

Figure 7. Structures of diglycerides.

DAG-1,2 Diacylglycerol; Ptd OH-Phosphatidic acid; CDP-DAG-Cytidine diphosphate diacylglycerol.

aggregatory agent and it has been suggested that lyso Ptd OH, rather than Ptd OH, may be an important intracellular messenger via an ionophoric activity (Benton et al., 1982). However not only is lyso Ptd OH unable to translate Ca^{2+} across liposomes (Lapetina & Watson, 1985) but they also stimulate platelets via a cell surface receptor (Watson et al., 1985a). It is therefore unlikely that lyso Ptd OH is a second messenger in agonist induced platelet activation.

3.6 Inositol phosphates

The formation of a Ca^{2+} mobilising second messenger must be extremely rapid as, by definition, it must precede the increase in $[Ca^{2+}]_i$. Berridge demonstrated the liberation of $Ins(1,4,5)P_3$ in the blowfly salivary gland within 5 seconds of challenge with 5HT (Berridge, 1983). The possibility of a second messenger role for $Ins(1,4,5)P_3$ was enhanced when, in permeabilised pancreatic acinar cells, $Ins(1,4,5)P_3$ was shown to release Ca^{2+} from an intracellular, non mitochondrial store (Streb et al., 1983). This has subsequently been demonstrated in a variety of tissues including hepatocytes (Burgess et al., 1984), vascular smooth muscle (Somlyo et al., 1985), skeletal muscle (Vergara et al., 1985) and platelets (O'Rourke et al., 1985; Authi & Crawford, 1985).

In addition $Ins(1,4,5)P_3$ induces platelet aggregation and 5HT release in a manner similar to thrombin (Authi et al., 1986; Watson et al., 1986). These effects, however, were abolished by cyclo oxygenase blockade indicating that $Ins(1,4,5)P_3$

induced Ca^{2+} mobilisation activates phospholipase A_2. The PG endoperoxides and TxA_2 generated as a result induce platelet activation via external receptors.

An intracellular $Ins(1,4,5)P_3$ acceptor has recently been identified in hepatocytes (Spat et al., 1986) and adrenal cortex (Baukal et al., 1986). Although $Ins(1,4,5)P_3$ is the most potent Ca^{2+} mobilising compound, other inositol phosphates, $Ins(2,4,5)P_3$ and $Ins(4,5)P_2$, both bind to the acceptor (Spat et al., 1986) and mobilise Ca^{2+} (Burgess et al., 1984). $Ins(1,4)P_2$, Ins 1P, Ins(1,2-cyc)P and $Ins(1,3,4)P_3$ neither bind to the acceptor nor mobilise Ca^{2+} thereby strengthening the correlation between binding and release.

The cyclic phosphate, $Ins(1,2\text{-cyc},4,5)P_3$, which has been reported to be the major product in thrombin stimulated platelets (Majerus et al., 1986) can also mobilise Ca^{2+} in permeabilised platelets (Wilson et al., 1985b). $Ins(1,2\text{-cyc},4,5)P_3$ is the only cyclic inositol phosphate possessing such activity, however the characteristics of its binding to the intracellular $Ins(1,4,5)P_3$ acceptor have not been reported.

Recently Putney suggested a model which proposed an action of $Ins(1,4,5)P_3$ in the agonist stimulated influx of Ca^{2+}. The "capacative" model proposes that $Ins(1,4,5)P_3$ activates the discharge of Ca^{2+} from its intracellular store, situated at the plasma membrane, into the cytosol. The decrease in the Ca^{2+} content of this pool relieves an inhibitory constraint on a direct pathway for extracellular Ca^{2+} to enter the pool. In the continued presence of $Ins(1,4,5)P_3$, Ca^{2+} will continue into the cytosol down the concentration gradient thereby

sustaining Ca^{2+} entry into the cell (Putney, 1986). Ins(1,3,4,5)P_4 may promote the rapid release of sequestered Ca^{2+} from liver plasma membranes (Hansen et al., 1986). It is possible that it is the formation of Ins(1,3,4,5)P_4, involving a metabolically expensive process, and not Ins(1,4,5)P_3 which is responsible for the translocation of Ca^{2+} across the plasma membrane of stimulated cells.

3.7 Diacylglycerol

The other major product of phosphoinositide hydrolysis is DAG which also has an important second messenger role in that it activates protein kinase C with the resultant protein phosphorylation which, in turn, has been implicated in platelet function (Takai et al., 1979, 1981a).

DAG is normally absent from membranes, but upon stimulation by agonists, very rapid increases have been measured in platelets (Rittenhouse-Simmons, 1979) and other cells (Berridge, 1984). The activation of protein kinase C by DAG is a complex process that requires Ca^{2+} and Ptd Ser as co-factors. It appears that relatively small amounts of DAG dramatically increases the affinity of protein kinase C for Ca^{2+} thereby fully activating the enzyme at resting $\left[Ca^{2+}\right]_i$ (Kishimoto et al., 1980). In the presence of DAG, protein kinase C becomes reversibly attached to membranes where it exerts its enzymatic activity. DAG is only transiently produced in membranes due both to its phosphorylation to Ptd OH and its degradation to liberate arachidonic acid. This transient appearance of DAG in membranes is always associated with activation of protein

kinase C (Huang & Detwiler, 1986a).

The activation of protein kinase C by DAG is mimicked by the tumour promoting class of compounds, the phorbol esters (Castagna et al., 1982). Phorbol esters, such as phorbol 12 myristate 13 acetate (PMA; 12 O tetradecanoylphorbol 13 acetate; Figure 8), have structural similarities to DAG (Figure 7) and are capable of causing platelet aggregation (Zucker et al., 1974) and release (White et al., 1974). Like DAG, phorbol esters increase the affinity of protein kinase C for Ca^{2+} resulting in enzymatic activity and platelet activation without detectable mobilisation of Ca^{2+} (Rink et al., 1983). Once again Ptd Ser appears to be a necessary co-factor (Nishizuka, 1984). In contrast to the transient nature of DAG accumulation, phorbol esters are metabolised very slowly and so activate protein kinase C for a prolonged period.

The use of phorbol esters (or of the synthetic DAG, 1 oleoyl 2 acetyl glycerol, OAG) in concert with Ca^{2+} ionophores has demonstrated a marked synergism in their abilities to elicit the physiological response (Yamanishi et al., 1983; Halenda et al., 1985). The balance of these two synergistic pathways, both emanating from phosphoinositide hydrolysis, may exert differential control over different processes within a single activated cell, such as the agonist selectivity observed for the release of platelet granular constituents (Nishizuka, 1984).

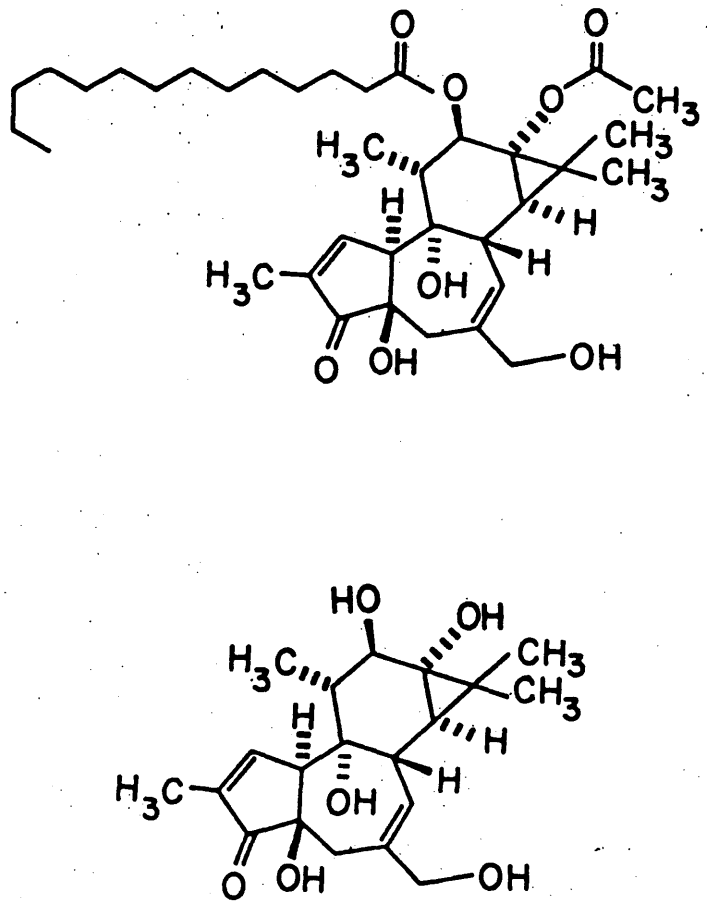

Figure 8. Structures of phorbol compounds.

Upper-Phorbol 12 myristate 13 acetate (PMA; active compound).
Lower-4β-Phorbol (inactive analogue).

3.8 Protein phosphorylation

Stimulation of platelets by thrombin is associated with the phosphorylation of a number of proteins. The two most readily observed have a M_r of 20,000 and a M_r of 40,000-47,000, termed 20K and 40K respectively (Lyons et al., 1975; Haslam & Lynham, 1977). In addition phosphorylation of proteins with M_r = 56,000 and 81,000 (Wallace & Bensusan, 1980), of actin binding protein (Carrol & Gerrard, 1982) and the delayed phosphorylation of a protein with M_r of 27,000 (Chambard & Pouyssegur, 1983) have all been reported. Most attention has, however, been given to the 20K and 40K proteins, phosphorylation of which correlates to secretion but not to aggregation (Haslam & Lynham, 1977).

The 20K protein has been identified as a light chain of myosin and its phosphorylation by a calcium calmodulin dependent protein kinase, as a consequence of elevated $[Ca^{2+}]_i$, enhances actin activated myosin ATPase activity (Adelstein & Conti, 1975). The 20K protein regulates the contractility of actomyosin and its phosphorylation parallels the association of myosin with cytoskeletal proteins in platelets (Huang & Detwiler, 1986a).

The 40K protein is one of the major substrates of protein kinase C and is phosphorylated when the enzyme is activated either by endogenous DAG or by phorbol esters (Castagna et al., 1982; Nishizuka, 1984). Recently it has been proposed that this protein has anti phospholipase A_2 activity and its phosphorylation removes this inhibitory constraint thereby mobilising arachidonic acid (Touqui et al., 1986). In addition a 40K protein has been isolated from stimulated platelets which

has been demonstrated to have Ins(1,4,5)P_3 phosphatase activity (Connolly & Majerus, 1986). Therefore, the 40K protein may not be a single moiety, but a combination of a number of active species each with a M_r of approximately 40K which have not yet been identified individually. Little is known of the functions of the other proteins which are phosphorylated in response to stimuli.

3.9 Arachidonic acid

Eicosanoids, such as PGG_2, PGH_2 and TxA_2, are secreted from platelets in response to agonists. It has been proposed that the agonist dependent hydrolysis of phosphoinositides plays a vital role in this release (Bell et al., 1979; Rittenhouse, 1982). The fatty acid on position of 2 of the phosphoinositides is normally arachidonate although oleate and linoleate may also be present. However during agonist stimulation phospholipase C activity is preferentially directed against those phosphoinositides containing arachidonate, such that all the DAG and Ptd OH formed has arachidonate in position 2 (Bell et al., 1979). Several mechanisms for the release of arachidonate have been proposed.

i) Direct deacylation of phospholipids by a phospholipase A_2 type enzyme (McKean et al., 1981; Bills et al., 1977).

ii) Deacylation by a phospholipase A_1 activity followed by a lysophospholipase.

iii) Sequential activation of Ptd Ins specific phospholipase C followed by DAG lipase and MAG lipase (Bell et al., 1979; Prescott & Majerus, 1983).

iv) Sequential activation of Ptd Ins specific phospholipase C, DAG kinase and Ptd OH specific phospholipase A_2 (Billah et al., 1981; Lapetina, 1982).

It is possible that eicosanoids serve as positive feedback second messengers in platelet activation particularly as cyclooxygenase inhibitors reduce, not only platelet responsiveness but also the hydrolysis of Ptd Ins, the formation of DAG induced by collagen (Rittenhouse & Allan, 1982) and the formation of Ptd OH induced by low concentrations of thrombin (Siess et al., 1983a).

3.10 cGMP

In platelets, in common with other cells (Michell, 1975), agonists which trigger phosphoinositide hydrolysis also stimulate guanylate cyclase activity to form cGMP (White et al., 1973; Haslam et al., 1980). The mechanism of this stimulation is unclear but seems to depend upon an increase in $[Ca^{2+}]_i$ (Berridge, 1984).

Haslam and McClenaghan (1974) proposed a role for cGMP in the platelet release reaction. Davies and colleagues (Davies et al., 1976) correlated cGMP levels with aggregation although probably as a consequence rather than as a cause. However 5HT and adrenaline elevate cGMP levels with no aggregation (Laubscher & Pletscher, 1980) and these authors suggested that cGMP generation was secondary to platelet

activation. The function of cGMP in platelet activation is further complicated by inhibitory effects of the guanylate cyclase stimulant sodium nitroprusside on platelet activation (Glusa et al., 1974; Levin et al., 1982), elevated $[Ca^{2+}]_i$ (Kawahara et al., 1984), DAG formation and 40K phosphorylation (Takai et al., 1981b) suggesting perhaps a negative feedback role for cGMP.

4. Summary

Figure 9 summarises the two transduction processes so far identified in the human platelet.

Stimulatory agonists (A_1) such as PAF, vasopressin, 5HT and TxA_2 combine with their specific cell surface receptors which are linked, possibly via a G protein, to phospholipase C. Activation of this enzyme results in the hydrolysis of one, or more, of the inositol phospholipids with the consequent production of DAG and a plethora of inositol phosphates. DAG, although produced only transiently, activates a Ptd Ser and Ca^{2+} dependent enzyme, protein kinase C. Protein kinase C in turn phosphorylates a protein(s) which has been implicated in platelet function. IP_3 ($Ins(1,4,5)P_3$ and/or $Ins(1,2\text{-cyc},4,5)P_3$) release Ca^{2+} from an intracellular, non-mitochondrial store, probably in the dense tubular system. This agonist induced increase in $[Ca^{2+}]_i$ is enhanced by an influx from the external milieu. The mechanism of this influx is unknown, although roles for Ptd OH, lyso Ptd OH, $Ins(1,4,5)P_3$ and $Ins(1,3,4,5)P_4$ have all been proposed. Regardless of the source of this Ca^{2+}, it activates a calcium calmodulin dependent protein kinase which

phosphorylates the light chain of myosin which plays a role in platelet stimulation and which may synergise with the DAG mediated activation.

The adenylate cyclase system is also active in the platelet. Inhibitory agonists (A_2), such as PGI_2, PGD_2 and adenosine, acting on their specific receptors, activate adenylate cyclase via the stimulatory G protein, N_s. This leads to elevated cAMP levels which in turn activate a cAMP-dependent protein kinase and the resultant protein phosphorylation inhibits platelet reactivity.

Whether the converse is true, that is decreased cAMP concentrations stimulates platelet function, as has been proposed for adrenaline, is the subject of controversy.

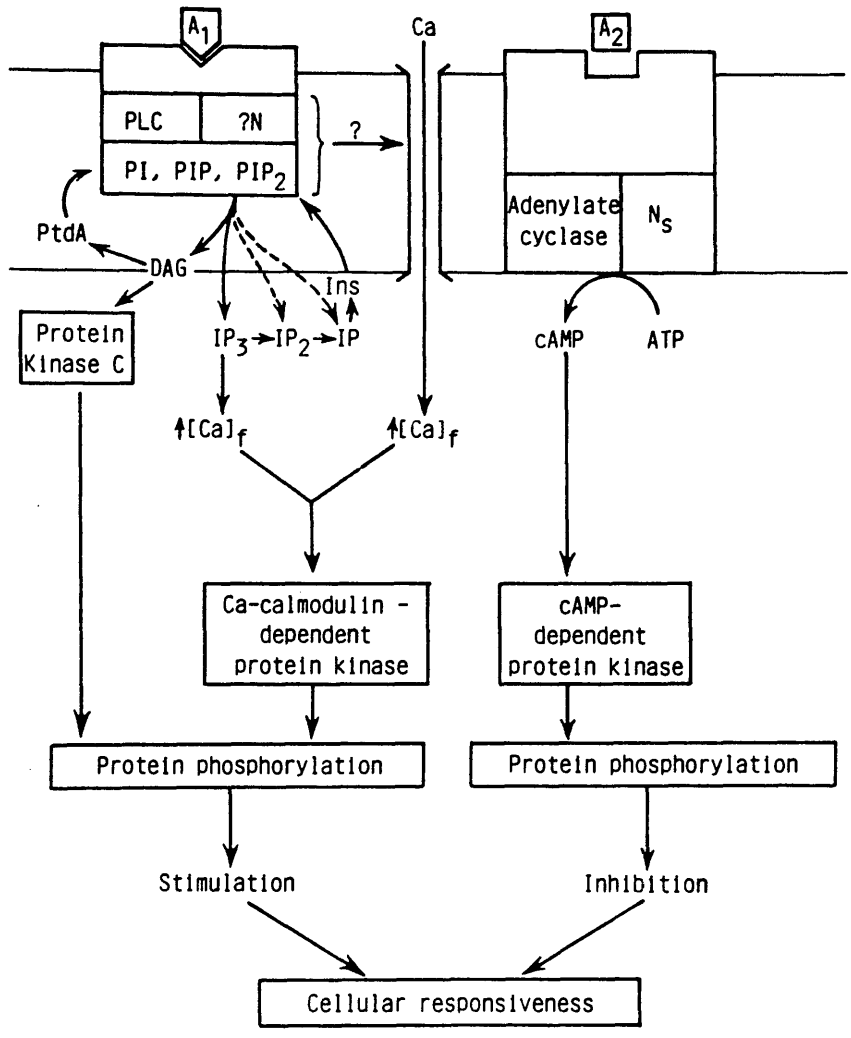

Figure 9. Transduction processes in the human platelet.

This is a summary of the transduction processes present in the human platelet. A_1 represents a stimulatory agonist, such as PAF, vasopressin or TxA_2 and A_2 represents an inhibitory agonist such as PGI_2 or PGD_2.

CHAPTER III: EXPERIMENTAL INDICES OF PLATELET REACTIVITY

There are a variety of measurements of platelet reactivity *in vitro* which allow the correlation to be made between cellular (functional) responses and various biochemical reactions.

1. Measurement of platelet functional responses to agonist exposure

1.1 Shape change

Platelet shape change *in vitro* is monitored photometrically. With the exception of adrenaline, addition of an agonist to a stirred suspension of either platelet rich plasma or washed platelets causes a decrease in the amount of light transmitted in the plane perpendicular to the axis of stirring. This occurs as a direct result of the projection of pseudopodia from the cell body. Shape change, unlike aggregation, occurs in the absence of calcium in the extracellular environment (Zucker & Zaccardi, 1964). Born (1970) utilised this differential dependency upon divalent cations between these two functions to monitor shape change independently of aggregation. Chelating agents, such as EDTA, when added to platelet preparations prevented the platelet shape change from progressing to aggregation, and therefore allowed the former response to manifest itself fully.

1.2 Adhesion

Measurement of the adhesive properties of platelets *in vitro* can be carried out by using the interaction between platelets and a glass surface. These employ a column of glass beads through which blood samples are passed and adhesiveness

estimated by the difference in platelet count before and after passage through the column (Hellem, 1960; Salzman, 1963). However, systems have now been devised which allow the exposure of natural vascular surfaces to blood in a controlled fluid dynamic environment, and the measurement of platelet adhesion by direct morphometric techniques (Baumgartner & Muggli, 1976; Sakariassen et al., 1983).

1.3 Aggregation

Aggregation is probably the most commonly measured platelet functional response. The standard method is a photometrical determination, which is simple and relatively inexpensive. Two novel approaches to the measurement of aggregation have been reported, both of which can be carried out in whole blood. Firstly it is possible to monitor the disappearance of single platelets, and secondly aggregation can be evaluated by an electronic method.

1.3(a) Photometric:

In 1962 a photometric method was introduced for the investigation of the aggregation of platelets (Born, 1962). A beam of light is directed through a suspension of platelets in plasma or in an artificial medium, the optical density of which is continuously recorded by measuring the transmitted light. The suspension is stirred at a rate sufficiently rapid to make the collision rate non limiting but insufficient to damage the cells. Agonist induced platelet aggregation causes the amount of transmitted light to increase and, conversely, when the aggregates disperse light transmission decreases.

This method has been the most frequently used, however it is limited by its sensitivity. It requires the formation of relatively large aggregates before platelet activation can be detected. It has been estimated that platelets challenged with ADP induce the removal of 87% of single cells with only 7% increase in light transmission (Born & Hume, 1967). In addition the modulatory actions of other blood cells, which may occur *in vivo*, cannot be readily monitored using this technique. This criticism can also be levelled against other methods adopted to monitor platelet aggregation in cell suspensions using both optical (Silverstein, 1976) and electronic (Gordon, 1973; Reuter & Deters, 1979) techniques.

1.3(b) Single cell disappearance:

A novel method for the estimation of single platelet disappearance has been reported (Day *et al.*, 1980; Lumley & Humphrey, 1981). This involves measuring the electrical impedance of platelets in whole blood which alters when a single platelet is removed (to form an aggregate). This method overcomes the limitations on the photometrical determination of platelet aggregation such as interaction of other blood cells and insensitivity.

1.3(c) Electronic measurement of aggregation:

Cardinal and Flower (1980) have also reported a method to monitor platelet aggregation in whole blood. A pair of electrodes are placed in a sample of blood and a small electric current passed between them. Platelets adhere to the electrodes, however in the presence of a stimulatory agonist the platelets

form aggregates around the electrodes and consequently impede the conductance. This method of measuring platelet aggregation has advantages over photometric determinations for the reasons outlined above.

1.4 Release

Secretion from human platelets can occur by two mechanisms. Firstly degranulation may occur, that is the selective release of storage granule contents. The second mechanism involves the synthesis and liberation of eicosanoids, which are not stored in their releasable form. The most common techniques used to measure the platelet release reaction involve detection of the dense granule constituents 5HT and ATP as an index of degranulation and measurement of TxA_2 formation as an index of stimulated synthesis of arachidonate metabolites.

1.4(a) 5HT:

5HT release can be monitored using isotope pre-labelling techniques (MacIntyre et al., 1978). Platelets are incubated with radiolabelled ($[^{14}C]$ or $[^{3}H]$) 5HT for a period of time which allows active uptake of the amine across the plasma membrane and incorporation of the label into the granules. The platelets are challenged with an agonist, the reaction terminated by appropriate means, 5HT re-uptake blocked pharmacologically, the platelets pelleted by centrifugation and a sample of the cell-free supernatant counted for presence of radiolabel. By inclusion of appropriate unstimulated controls and measurement of intracellular radiolabel, the extent

of stimulus-induced release of 5HT can be assessed.

1.4(b) ATP:

ATP release is routinely monitored using luciferin luciferase luminescence. The principle underlying this method is the interaction of the released ATP with synthetic luciferin and luciferase extracted from firefly tails to emit a luminescence signal (Detwiler & Feinman, 1973). This signal can be readily detected by means of a Photomultiplier Tube. The release of ATP can be quantified using standards or compared in extent to release elicited by a powerful agonist (e.g. 1U/ml thrombin). The extent of ATP secretion evoked by this agonist is defined as 100% of maximum releasable ATP. In addition this method allows for simultaneous measurement of agonist induced ATP release and aggregation. However evidence suggests that luciferin-luciferase may inhibit agonist induced aggregation (Thompson & Scrutton, 1985).

1.4(c) Thromboxane A_2:

Thromboxane A_2, the major eicosanoid produced by stimulated platelets, is extremely unstable and consequently it is quantified as the formation of its stable metabolite, TxB_2. Multiple ion analysis, bioassay, radioimmunoassay, gas chromatography/mass spectrography, HPLC and thin layer chromatography have all been used to measure TxB_2. In addition stimulation of platelets preincubated with radio labelled arachidonic acid, can be used as an estimation of cyclo oxygenase activity.

2. Measurement of platelet biochemical responses: Monitoring intracellular calcium levels

It is well accepted that calcium plays a pivotal role in the stimulation of platelets by a number of agonists (Gerrard et al., 1981). Although agonist induced calcium fluxes have been readily monitored using $^{45}Ca^{2+}$, a direct measurement of intracellular free calcium concentration ($[Ca^{2+}]_i$) and changes in $[Ca^{2+}]_i$ that result from exposure to agonists have not been possible until relatively recently.

2.1 Quin 2

$[Ca^{2+}]_i$ can be measured fluorimetrically using the Ca^{2+} indicator dye, 2 methyl 6 methoxy 8 nitroquinoline (Quin 2), trapped in the cytosol (Tsien, 1981). Quin 2 itself does not readily cross cellular membranes although the esterified form, Quin 2 acetoxy methylester (Quin 2 AM) is membrane permeant. Consequently incubation of cells with Quin 2 AM results in incorporation into intact cells, cleavage by cytoplasmic esterases to the parent Quin 2 which is effectively trapped in the cytosol. Such a procedure does not involve disruption of the cellular membrane. Quin 2 is a calcium chelator which in the presence of free Ca^{2+} displays a fluorescence peak at 492 nm emission when excited at 339 nm. The measured fluorescence levels can be calibrated for $[Ca^{2+}]_i$ by exposing the dye to known Ca^{2+} concentrations at the end of each experiment. This is normally achieved by lysing the cells and titrating the Ca^{2+} in the lysate with the familiar chelator EGTA (Tsien et al., 1984). Quin 2 has a dissociation constant (115 nM) comparable

to typical resting $[Ca^{2+}]_i$ values near 100 nM and so is highly sensitive to variations near those levels. Quin 2 has three major drawbacks:

i) Quin 2 itself is a calcium chelating agent and so may mask the small, and underestimate the larger, $[Ca^{2+}]_i$ changes which may occur in unloaded cells. This problem is exacerbated as intracellular concentrations of Quin 2 must be in the millimolar range (Tsien et al., 1984).

ii) The suggested Kd for calmodulin is 2.5 μM (Rasmussen & Barrett, 1984), however Quin 2 is relatively insensitive to $[Ca^{2+}]_i$ at this concentration level.

iii) Quin 2 reports the spatial average $[Ca^{2+}]_i$ in the cell cytosol and thus is insensitive to small localised changes in specific intracellular calcium pools (Johnson et al., 1985).

Tsien and colleagues have recently developed a novel fluorescent indicator dye, Fura 2. The principle underlying Fura 2 is stimilar to that of its predecessor, however it exhibits a much greater fluorescence than Quin 2, undergoes a shift of emission maximum upon Ca^{2+} binding and may be used at lower (μM) loading concentrations in the cell (Grynkiewicz et al., 1985).

2.2 Aequorin

The use of large, calcium sensitive photoproteins, such as aequorin, in platelets was, until recently, impossible as they required to be administered to cells by micro-injection. However a technique has been developed which apparently allows platelets to be permeabilised, aequorin introduced into their

cytoplasm and then resealed (Johnson et al., 1985). The range of sensitivity of aequorin is 100 nM to 1 mM and its Kd is 3 μM, approximately the same as that of calmodulin (Salzman et al., 1985). In addition, there is evidence that aequorin is distributed inhomogeneously within cells. Consequently aequorin signals may represent local elevations in $[Ca^{2+}]_i$, such as might be expected to occur in the early stages of a response to an agonist. This may also account for changes in $[Ca^{2+}]_i$ being monitored in platelets challenged with adrenaline or phorbol esters in studies using aequorin but not Quin 2 (Salzman et al., 1985).

3. Measurement of platelet biochemical responses: Monitoring the phosphoinositide cycle

The hydrolysis of the inositol phospholipids plays a central role in the activation of platelets by stimulatory agonists. A variety of techniques have evolved which permit the metabolic pathways involved to be studied. In general, two methods exist, firstly the use of radioactive tracers and, secondly the direct measurement of the moieties involved in the phosphoinositide cycle. The benefits and limitations inherent in each method are discussed below.

3.1 Radioactive labelling

Protocols adopted for the labelling of the inositol containing constituents in platelets have to be a balance between conditions of maximum incorporation of the isotope and those which are conducive to the survival of the cells in vitro. In the former case optimal labelling conditions

involves the suspension of the platelets in a physiological buffering solution and incubation with the isotope for a period of time to allow the label to be incorporated into the appropriate intracellular constituent to be examined. In the latter case, platelets outwith the corporeal circulation have a limited life-span, even when conditions are propitious to their survival. Specifically, agonist induced phosphoinositide hydrolysis can be monitored by measuring:-

1) a decrease in the levels of the parent inositol phospholipid; Ptd Ins, PIP or PIP_2.

2) the formation of the products of hydrolysis; DAG or inositol phosphates.

3) an increase in the turnover of the phosphoinositide cycle as a whole.

3.1(a) ^{32}P incorporation:

The incorporation of $[^{32}P]$ orthophosphate (P_i) into Ptd Ins and Ptd OH remains the most widely used, indirect method of monitoring phosphoinositide turnover. Ptd Ins labels with $[^{32}P]$ P_i rapidly as, unlike other phosphatidate esters (e.g. Ptd Cho), the phosphate group is derived directly from Ptd OH (Figure 10). Ptd OH in turn gains the ^{32}P label from the gamma, or terminal, phosphate group of cellular ATP. When short term labelling conditions (1-2 hours) are employed, after which excess label is removed, the pool of $[^{32}P]$ Ptd OH is in isotopic equilibrium with the cellular $[^{32}P]$ ATP (Holmsen et al., 1984) and so changes in label reflect changes in Ptd OH concentrations. In contrast, due to the relatively large

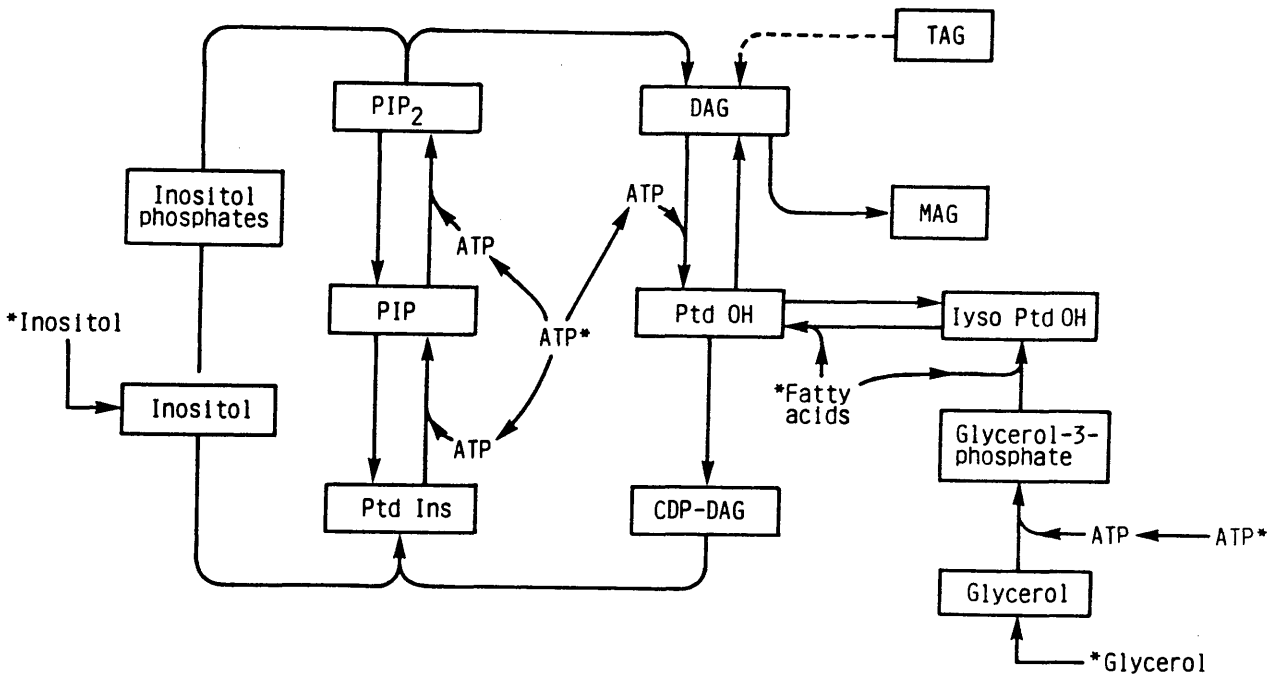

Figure 10. Incorporation of radiolabels into phosphoinositide cycle.

The radio-labels which are routinely used to monitor inositol phospholipid hydrolysis are indicated by *.

cellular content of Ptd Ins, it would require several hours incubation with $[^{32}P]P_i$ to label $[^{32}P]$ Ptd Ins to equilibrium with the $[^{32}P]$ ATP. Although this can readily be achieved using cells grown in culture, it is impossible to achieve such an equilibrium without radically impairing the functional viability of platelets.

The polyphosphoinositides, PIP and PIP_2, are derived from Ptd Ins and their 4- and 5-phosphate groups, which turn over extremely rapidly (Hawthorn & Pickard, 1979), are donated directly from the pool of ATP (Figure 10). In addition to this rapid turnover, the polyphosphoinositides are present in minimal amounts and so the 4- and 5-phosphate groups quickly achieve an equilibrated state with the cellular $[^{32}P]$ ATP.

Agonist receptor interaction results in the phospholipase C mediated hydrolysis of the inositol phospholipids, resulting in the formation of DAG which is rapidly phosphorylated to Ptd OH by DAG kinase. In labelled platelets, where $[^{32}P]$ Ptd OH is at equilibrium with $[^{32}P]$ ATP, this manifests itself initially as an increase in $[^{32}P]$ Ptd OH. In addition $[^{32}P]$ Ptd Ins levels are also increased due to conversion of $[^{32}P]$ Ptd OH produced to $[^{32}P]$ PtdIns. These increased levels of $[^{32}P]$ -Ptd OH and -Ptd Ins have been assumed to be indicative of an accelerated turnover of the phosphoinositide cycle.

There are however limitations on studies involving $[^{32}P]P_i$ labelling (Irvine *et al.*, 1982).

i) The increase in $[^{32}P]$ -Ptd OH and -Ptd Ins levels are both secondary effects following the initial hydrolysis of

inositol phospholipids. Ptd OH formation is dependent upon DAG formation, and in turn Ptd OH is the precursor of Ptd Ins.

ii) Although phospholipase C activity, via the formation of DAG, is believed to be the major source of Ptd OH, another two minor mechanisms may affect $[^{32}P]$ Ptd OH formation independent of the phosphoinositide cycle. Stimulation of triglyceride lipase will also elicit DAG formation (from TAG), however this enzyme is reportedly absent from human platelets (Lote & Lowery, 1979). De novo synthesis of Ptd OH can also occur by the acylation of glycero 3 phosphate to lyso Ptd OH. This is a relatively slow pathway (Neufield & Majerus, 1983) and so is not believed to influence the early accumulation of $[^{32}P]$ Ptd OH which follows agonist-induced platelet activation.

It is assumed therefore that increased $[^{32}P]$ Ptd OH levels reflect an increased Ptd OH mass and that stimulation of $[^{32}P]$ -Ptd OH and -Ptd Ins are indicative of activation of phosphoinositide hydrolysis.

3.1(b) Glycerol labelling:

Glycerol is incorporated into the backbone of phospholipids by its conversion to glycerol 3 phosphate which is the common precursor for all phospholipids (Figure 10). $[^{3}H]$ glycerol labelling has been extensively used to monitor the phosphoinositide cycle and allows the measurement of the initial hydrolysis of $[^{3}H]$ inositol phospholipids without rapidly altering their specific activity. Such changes can be monitored when the isotopic labelling has not reached equilibrium, however the large amounts of endogenous DAG and Ptd OH formed

may mask the corresponding increases in $[^3H]$ -DAG and -Ptd OH. $[^3H]$ glycerol labelling is often used to verify studies using $[^{32}P]P_i$, however such studies are compromised by the differing rates of incorporation of the labels (Irvine et al., 1982).

3.1(c) Fatty acid labelling:

Radioactive fatty acids are also used to label the backbone of phospholipids as they are esterified in position 1 and 2 of the glycerol backbone (Figure 10). $[^3H]$ or $[^{14}C]$ arachidonic acid is preferentially used as it specifically reacylates the phosphoinositides at position 2. This label has three principal limitations (Irvine et al., 1982).

i) Stimulated phospholipase A_2 activity causes the liberation of labelled arachidonic acid metabolites. Such activity may lead to an over estimation of Ptd Ins breakdown and an under estimation of the levels of DAG and Ptd OH formed in response to agonists.

ii) DAG lipase activity removes arachidonic acid from DAG with the resultant decrease in the observed levels of labelled Ptd OH.

iii) Specific reacylation may occur, which results in a change in the specific activity of the lipids. This change would not reflect changes in the mass of the lipids and so would complicate such studies.

3.1(d) Inositol labelling:

$[^3H]$ inositol can be used to measure activation of the phosphoinositide cycle by both indirect and direct methods. Indirectly phosphoinositide turnover is measured by an increased

incorporation into the phospholipids in a manner similar to $[^{32}P]P_i$ labelling and so is subject to similar restrictions. Inositol labelling allows the agonist induced accumulation of $[^3H]$ inositol phosphates to be monitored directly (Figure 10). These water soluble compounds can be readily separated (e.g. on Dowex columns; Berridge et al., 1983) and effectively allows simultaneous measurement of inositol lipid hydrolysis and inositol phosphate production.

The tissue inositol content is in excess of the extracellular levels (Dawson & Freinkel, 1961) and hence passive uptake of $[^3H]$ inositol is limited. Therefore under conditions where the viability of the platelet is maintained, unless prohibitively expensive quantities of $[^3H]$ inositol are utilised (Watson et al., 1984, 1985b) there is insufficient labelling to ensure phosphoinositide hydrolysis can be accurately measured.

3.2 Measurement of phosphoinositide amounts

The artifacts associated with labelling studies to monitor phosphoinositide metabolism can be avoided by direct measurement of the various constituents of the phosphoinositide cycle. This can be achieved either by measuring the amount of phospholipid present, normally by the chemical analysis of the inorganic phosphorous (P_i) present, or by monitoring the formation of the inositol phosphates, either by HPLC or by gas chromatography.

3.2(a) Inorganic phosphorous assays:

A large number of assays for the determination of P_i have been reported and a selection are shown on Table 2 along with the level of detection claimed by the authors. Each assay depends upon the release of P_i from the phospholipid, normally achieved by digesting the lipid with an acid, such as perchloric. The basis of each of the assays is a reaction between the released P_i and ammonium molybdate to liberate phosphomolybdic acid which in turn is reduced and the product quantified spectrophotometrically. The method of Bartlett (1959) was the method of choice for a large number of studies and has been modified and the sensitivity improved (Usher, 1963; Christie, 1982). This method utilises a mixture of sodium bisulphite, sodium sulphite and 1 amino 2 napthol 4 sulphonic acid as a reducing agent. Bartlett claimed that the original method was sensitive enough to measure 150 nmoles of P_i. Usher's version measured 16 nmoles and Christie claimed that his protocol was more sensitive without quoting the limits of the assay. The methods of Itaya and Ui (1966), Hess and Derr (1975) and Bowyer and King (1977) reduced the phosphomolybdic acid using malachite green and had sensitivities of 1.6, 1.0 and 1.6 nmoles of P_i respectively. Bowyer and King detailed their protocol specifically for the measurement of the P_i content of phospholipids separated by thin layer chromatography. Similarly Rouser and his colleagues (1969) reported a method of measuring mass amounts of phospholipid by quantification of their P_i content. The reducing agent in this case was ascorbic acid and the minimum level of detection was reported

REFERENCE	DETECTION LIMIT
Bartlett (1959)	150.0
Usher (1963)	16.0
Christie (1982)	N.D.
Itaya & Ui (1966)	1.6
Hess & Derr (1975)	1.0
Bowyer & King (1977)	1.6
Rouser et al. (1970)	0.1

Table 2. Assays for the measurement of inorganic phosphorous levels.

A list of assays which have been used to measure inorganic phosphorous levels and the resolution claimed by the authors (nmoles of inorganic phosphorous).

N.D. - not determined.

to be 0.1 nmoles P_i, by far the most sensitive assay of those listed.

There are, of course, several drawbacks using P_i assays to measure levels of phospholipids. Firstly, assays for P_i are tedious, time consuming and difficult to perform accurately. Secondly, there is relatively little P_i associated with phospholipids, and so the technique employed is required to be highly sensitive to avoid large tissue samples being necessary. Thirdly, the methods for the determination of P_i are subject to contamination which interferes with the assay. Such contamination can be overcome, however this requires measures which are expensive in terms of both time and cost.

3.2(b) Inositol phosphate assays:

Inositol phosphates, isolated in acidic conditions can be monitored by HPLC (Irvine et al., 1985) and by using a combination of ion exchange and capillary gas chromatography (Rittenhouse and Sasson, 1985).

These procedures are advantageous over those involving [^{3}H] inositol prelabelling as they overcome the various labelling artifacts involved. In addition both can be readily used for cells, including platelets, where the incorporation of inositol is limited. Although various isomers of the inositol phosphates can be readily identified, these methods, in common with prelabelling studies, involve an acidic extraction procedure and so any cyclic phosphates formed would be hydrolysed to the linear form.

CHAPTER IV: DESENSITISATION AND POTENTIATION

1. Desensitisation

It is well known that many cells possess mechanisms which allow them to regulate their responses to external stimuli. Desensitisation (also described as tachyphalaxis, tolerance, fade, refractoriness, subsensitivity) is the loss of cellular sensitivity during, or subsequent to, the initial action of a drug (Triggle, 1980). This desensitisation is an ubiquitous occurrence in systems as diverse as bacterial organisms and mammalian tissues and suggests that this phenomenon represents an important regulatory component of the homeostatic capacity of the cellular recognition process. Two distinct types of desensitisation have been recognised. The term "homologous" desensitisation was applied to designate the type of desensitisation observed when cells were rechallenged with the same agonist used in the initial exposure; "heterologous" desensitisation referred to the situation where re-exposure was to an alternative agonist (Su *et al.*, 1976). The mechanism (s) underlying both forms of desensitisation have been the subject of much speculation. A variety of potential mechanisms, including alterations in receptor structure and number, in the membrane lipid environment and the inhibition or uncoupling of transduction processes, have been postulated and several of these are discussed below.

1.1 Platelet desensitisation

Platelet functional responses, normally aggregation, display the phenomenon of homologous desensitisation. ADP (O'Brien, 1966; Evans & Gordon, 1974; Ruggles & Scrutton, 1979), 5HT (Baumgartner & Born, 1968; Evans & Gordon, 1974; Ruggles & Scrutton, 1979), vasopressin (Ruggles & Scrutton, 1979), thrombin (Ruggles & Scrutton, 1979; McGowan & Detwiler, 1983), adrenaline (Motulsky et al., 1986) and TxA_2, using stable analogues (MacIntyre, 1981), all exhibit this phenomenon. The possibility of heterologous desensitisation is unclear. Prior exposure of platelets to ADP renders them less responsive to collagen (Evans & Gordon, 1974; Ruggles & Scrutton, 1979) but those responses to adrenaline (O'Brien, 1966), 5HT (Baumgartner & Born, 1968), thrombin and vasopressin (Ruggles & Scrutton, 1979) are unaffected. Addition of 5HT has been reported to desensitise platelets to ADP and adrenaline (Baumgartner & Born, 1968) although this has been disputed (Evans & Gordon, 1974; Ruggles & Scrutton, 1979). In addition Ruggles and Scrutton reported that platelets challenged with 5HT and thrombin become refractory to collagen and in the case of thrombin also to vasopressin and arachidonic acid (Ruggles and Scrutton, 1979). With the exception of the above cases, ADP, adrenaline, thrombin, 5HT and vasopressin have been reported to be ineffective at inducing heterologous desensitisation (Ruggles & Scrutton, 1979; Motulsky et al., 1986). Partial agonists at the ADP or α-adreno receptors do not induce significant desensitisation to the subsequent addition of a full agonist (Ruggles & Scrutton, 1979).

Studies into the role of second messengers in the development of platelet desensitisation have been limited to the adenylate cyclase system. Adrenaline induces homologous desensitisation without affecting the ability of the α_2 receptors to inhibit adenylate cyclase (Motulsky et al., 1986). The platelet inhibitory agonists PGI_2 and PGD_2 cause homologous but not heterologous desensitisation of adenylate cyclase (Miller & Gorman, 1979). In contrast to platelets, a large number of studies in a variety of other cell types have addressed the role of various transduction processes in the mechanisms underlying desensitisation. The vast majority of these reports have involved an investigation of desensitisation in the adenylate cyclase system.

1.2 Adenylate cyclase

The β adrenergic receptor linked adenylate cyclase system has provided a model system for the study of the mechanisms underlying desensitisation. Kakiuchi and Rall (1968) were first to demonstrate that following the exposure of a tissue (rabbit brain slices) to an agonist (adrenaline or histamine) there would be a reduction in the accumulation of the second messenger (cAMP) with time. In this study, desensitisation was limited to the agent used in the first exposure. Perkins and colleagues (Su et al., 1976) extended these findings in human astrocytoma cells, where short term incubations with either catecholamines or PGE_2 resulted in desensitisation to a subsequent addition of the same agonist. In contrast, if the cells were incubated for an extended period with a

catecholamine, there was a diminished response to PGE_2, and vice versa. Therefore Perkins proposed the two distinct forms of desensitisation now accepted. Heterologous desensitisation was slower occurring and less extensive than homologous desensitisation. Furthermore this heterologous desensitisation correlated to cAMP formation and it was proposed to occur through a cAMP mediated reduction in adenylate cyclase activity. Homologous desensitisation was thought to be comprised of both the cAMP mediated phenomenon that occurs during heterologous desensitisation and an agonist specific phenomenon that is unrelated to the elevation of cAMP (Harden, 1983). Antagonists can block homclogous but not heterologous desensitisation. In addition a good correlation is observed between efficacy for adenylate cyclase stimulation and the extent of desensitisation induced, i.e. partial agonists induce "partial desensitisation". This is presumably indicative of an intimate relationship between receptor occupancy, and therefore activation of the transduction process and formation of second messengers, and the generation of desensitisation.

1.2(a) Heterologous desensitisation:

Investigation into the mechanism of heterologous desensitisation of the adenylate cyclase system has centered on the post receptor events. In a variety of adenylate cyclase mediated cells, the role of cAMP in desensitisation has been supported by the use of cAMP analogues (Terasaki et al., 1978), cholera toxin (Nickols & Brooker, 1980) and phosphodiesterase

inhibitors (Terasaki et al., 1978) all of which induce desensitisation. To date the component or process in the adenylate cyclase system regulatory cycle that is modified during the process of desensitisation is unclear. However several possible mediators have been ruled out:-

1) Destruction of the agonist during stimulation

2) Increased egress of cAMP into the extracellular medium

3) Increased phosphodiesterase activity.

Desensitisation is unaffected by protein synthesis inhibitors, with the exception of rat glioma cells. In this tissue desensitisation is markedly reduced in the absence of protein synthesis and, a so far unidentified, mediator of desensitisation has been proposed. Likewise, fat cells are unique during the development of desensitisation. Stimulation by adrenaline, ACTH or glucagon all cause cellular desensitisatio accompanied by the formation of an inhibitor of agonist action. The appearance of this "feedback regulator" correlation to cAMP formation and is also stimulated by dibutyrl cAMP.

Studies in red blood cells, of various species, demonstrated that in these cells desensitisation is stable to cell lysis and likely involves a covalent modification of the G proteins (Harden, 1983). This modification could result in G proteins being less efficient promoters of catalytic activity, as proposed for fibroblasts (Kassis & Fishman, 1982), and/or an alteration in the rate of exchange of GTP for GDP on the G proteins, as proposed for pigeon erythrocytes (Hudson & Johnson, 1981). In general however heterologous desensitisation is

not observed in membrane preparations and so may involve a process dependent on cellular integrity (Triggle, 1980). Therefore there is probably no universal mechanism for heterologous desensitisation of the adenylate cyclase system.

1.2(b) Homologous desensitisation:

In contrast to heterologous desensitisation, in all systems studied the phenomena of homologous desensitisation has been attributed to a common mechanism.

In frog cells, the desensitising action of catecholamines can be blocked, or reversed, by propranolol. This indicates that desensitisation is caused by a reduction in the number of functional receptors, termed down regulation. It is likely that chronic occupancy, rather than active loss of the receptors, is responsible as receptor number and adenylate cyclase activity is unaffected by protein synthesis inhibitors (Mukherjee et al., 1976). Labelling studies, in erythrocytes, demonstrated that agonists dissociate from receptors much slower than antagonists and this dissociation is accelerated by guanyl nucleotides (Williams & Lefkowitz, 1977). Antagonists are ineffective at inducing, and guanyl nucleotides reverse, desensitisation (Mukherjee & Lefkowtiz, 1976), and this is further evidence for prolonged receptor occupancy mediating acute desensitisation. In studies involving erythrocytes, Shear and colleagues (Shear et al., 1976) concluded that this receptor occupancy alone or occupancy in the presence of elevated cAMP levels was insufficient for the agonist induced down regulation of receptors. Several studies have demonstrated that for receptor down regulation to occur, agonist induced coupling of the receptor to G proteins

is mandatory although the presence of adenylate cyclase, or the formation of cAMP, is not (Harden, 1983). It is now believed that during desensitisation the G proteins remain unaltered and it is the receptor subunit which is modified (Iyengar et al., 1981). The nature of this alteration in the β adrenoceptor is unknown although phosphorylation/dephosphorylation, disulphide-sulphydryl exchange, methylation/demethylation or receptor proteolysis have all been proposed to occur in a variety of cells (Hollenberg, 1985a). The chronic effects of receptor occupancy may well be the clustering and internalisation of the receptors with the concomitant loss of receptors over a period of hours (Su et al., 1979, 1980). Thus a pathway for homologous desensitisation would involve the interaction of an agonist with its cell surface receptor and the resultant activation of adenylate cyclase via a G protein. This receptor is then rapidly converted to a form no longer coupled to the G proteins. These desensitised, uncoupled receptors are then rapidly clustered and internalised and become undetectable to radioligand binding.

1.3 Calcium mediated systems

Complex patterns of desensitisation can also be observed in non adenylate cyclase linked systems. However the mechanisms involved in such systems are less well documented.

The rat parotid gland is one of the few tissues in which desensitisation of the Ca^{2+} signalling system has been studied. Homologous desensitisation of α adrenergic induced potassium release (Strittmatter et al., 1977) and α-amylase secretion

(Harper & Brooker, 1978) has been observed and attributed to an effect at the receptor level. However Putney demonstrated that not only does the K^+ efflux stimulated by carbachol or substance P fade with time, but a subsequent addition of an agonist, either the same or different to the original, fails to produce a response (i.e. heterologous desensitisation) (Putney, 1978). It was further suggested that this desensitisation was mediated by a Ca^{2+} influx and may be due to a development of insensitivity of the K^+ channel for activation by elevated $[Ca^{2+}]_i$, rather than an effect at either the receptor or Ca^{2+} channel.

Studies in guinea pig pancreatic acinar cells have shed light upon the role of interactions between second messenger systems in the development of desensitisation. In this tissue both increased cAMP levels and elevated $[Ca^{2+}]_i$ cause enzyme secretion. Heterologous desensitisation is observed when maximal concentrations of secretagogues that both elevate $[Ca^{2+}]i$ or both stimulate cAMP are used. This phenomena is not observed if two secretagogues with differing modes of action are used (Gardner & Jensen, 1980).

Desensitisation of Ca^{2+} gating has been observed in 5HT stimulated blowfly salivary glands (Berridge & Fain, 1979). This effect was attributed to a depletion of the phosphoinositides, which serve as the transduction process. The agonist was reported not only to stimulate the hydrolysis, but also inhibit the resynthesis, of the phospholipids. The possibility was also entertained that clustering of desensitised

receptors would limit their access to the remaining inositol phospholipids in the membrane. The addition of inositol, but not of choline or ethanolamine, to desensitised cells led to a complete recovery of responsiveness (Fain & Berridge, 1979).

The guinea pig ileum responds to a variety of smooth muscle stimulants, including acetylcholine, histamine and substance P, to generate a mechanical contraction mediated by an apparently common influx of Ca^{2+} from the extracellular space. Both homologous and heterologous desensitisation can be observed in this tissue. A major component to heterologous desensitisation is probably the hyperpolarisation of the cellular membrane caused by electrogenic pumping of the Na^{+} ions that enter the smooth muscle cells during the stimulation. This hyperpolarisation will increase the threshold for cellular excitation and render the cell less sensitive to stimulation (Triggle, 1980).

2. Potentiation

The second receptor mediated alteration which allows cells to regulate their response to the external environment is termed potentiation. The definition of potentiation (also called synergism) is often not clear although, in general terms, it is that situation where the response to the co-addition of two agonists is greater than the sum of the responses induced by either agonist acting alone. Potentiation has been less extensively studied than desensitisation.

2.1 Platelet potentiation

The potentiation of platelet aggregation has been widely reported. ADP synergises with collagen (O'Brien, 1964), thrombin (Niewiarowski & Thomas, 1966), adrenaline (O'Brien, 1964; Ardlie et al., 1966; Mills & Roberts, 1967) and 5HT (O'Brien, 1964; Baumgartner & Born, 1968; Michal & Motamed, 1976). In addition adrenaline potentiates responses elicited by collagen (Mills & Roberts, 1967), thrombin (O'Brien, 1964; Thomas, 1967; Steen & Holmsen, 1985), 5HT (Baumgartner & Born, 1968; Mills & Roberts, 1967) and PAF (Fouque & Vargaftig, 1984). The underlying biochemical mechanism of these potentiation effects have not been addressed in any of these studies.

2.2 Second messenger potentiation

Few studies have been reported which specifically investigate the potentiation of the production of second messengers. As outlined earlier, in guinea pig pancreatic acinar cells stimulated formation of cAMP and elevation of $[Ca^{2+}]_i$ both elicit enzyme secretion. If the cells are challenged with two agonists which have the same mode of action then the enzyme secretion is equal to that of the more effective secretagogue. In contrast however if an adenylate cyclase stimulant and a calcium mediated agonist are combined then there is a marked synergism in the effect of the agonist (Gardner & Jensen, 1980).

3. Pharmacology of desensitisation and potentiation

Many pharmacological effects can manifest themselves as changes in the position of the agonist concentration response curves. However there is an increasing reluctance to relate these changes to events at the molecular level. In general, receptor down-regulation will lead to a rightward shift of the concentration response curve. Since prolonged receptor occupation leads to down-regulation it may be anticipated that the curve will continuously shift to the right with time (Hollenberg, 1985b).

Desensitisation, following repeated administration of the agonist is indicated not only by a shift to the right in the concentration response curve (due to receptor down-regulation) but also by a reduction in the maximal obtainable response (Hollenberg, 1985b). The reverse, that is a leftward shift and increased maximal response, occurs when either agonists are potentiated or the cells are hypersensitive (Hollenberg, 1985b).

CHAPTER V: OBJECTIVES AND BACKGROUND OF STUDY

The general objectives of the study can be summarised as follows:-

i) To investigate the function, if any, of phosphoinositide hydrolysis in the mechanism of action of agonists which, hitherto, had not been studied.

ii) To employ new techniques to verify phosphoinositide hydrolysis as the mechanism of action of agonists.

iii) To further study the pharmacological profile of agonists such as the TxA_2 analogues and thrombin.

iv) To study possible biochemical mechanisms underlying the phenomenon of desensitisation.

v) To examine the role, if any, of phosphoinositide hydrolysis in the potentiation by ADP and adrenaline of platelet responses.

1. Effects of agonist on phosphoinositide hydrolysis

It is now generally accepted that the hydrolysis of one or more of the inositol phospholipids, with the consequent production of DAG and elevation of $\left[Ca^{2+}\right]_i$, serves as a transduction process in the stimulation of human platelets (Rink & Hallam, 1984). A variety of techniques have been employed to monitor phosphoinositide hydrolysis and have been used to elucidate the mode of action of a number of agonists. Thrombin, PAF, collagen, arachidonic acid, vasopressin, 5HT and TxA_2 (U44069) have all been shown to cause the breakdown of the phosphoinositides in human platelets (Agranoff *et al.*, 1983; MacIntyre & Pollock, 1983; Watson *et al.*, 1985b; Siess

et al., 1983b, 1986; Pollock, 1984; Pollock et al., 1984). Previous studies in the laboratory have contributed to, and extended, these findings and allowed the correlation between $[^{32}P]$ Ptd OH formation and elevation of $[Ca^{2+}]_i$ to be made. Figures 11 and 12 summarise these observations. PAF, U44069, vasopressin and 5HT, but not adrenaline, stimulate the formation of Ptd OH and elevation of $[Ca^{2+}]_i$. The first objective of this study was to complete this correlation by examining the effects of thrombin and ADP, both of which elevate $[Ca^{2+}]_i$, on $[^{32}P]$ phosphoinositide levels. In addition the novel TxA_2 mimetic EP171, reputedly the most potent such compound (Jones et al., 1985), was examined, as was the phorbol ester and protein kinase C stimulant, PMA (Castagna et al., 1982). In each case the levels of $[^{32}P]$-PIP_2, -PIP and -Ptd OH was monitored in $[^{32}P]$ P_i prelabelled human platelets.

2. Further analysis of U44069 and thrombin induced phosphoinositide hydrolysis

Thrombin is the most frequently used platelet agonist and is the most potent and efficacious stimulant of platelet functional and biochemical responses. Thrombin has been reported to decrease levels of the phosphoinositides (Broekman et al., 1980; Agranoff et al., 1983; Rendu et al., 1983) and correspondingly increase DAG (Rittenhouse-Simmons, 1979), Ptd OH (Broekman et al., 1980) and the inositol phosphates (Agranoff et al., 1983; Siess & Binder, 1985; Rittenhouse & Sasson, 1985). However, although a high affinity thrombin binding site on the platelet membrane correlates, at least partly, to cellular activation,

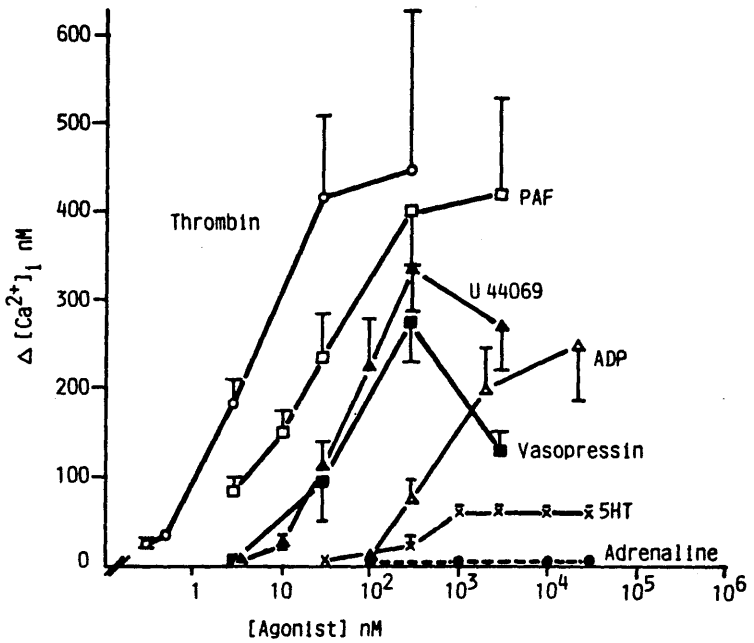

Figure 11. Agonist induced elevation of $[Ca^{2+}]_i$ in human platelets.

Agonists, at the concentration indicated, were added to Quin 2 labelled platelets suspended in a medium of external free Ca^{2+} = 1 mM. Changes in $[Ca^{2+}]_i$ were calculated from the observed changes in dye fluorescence (Figure 2 in MacIntyre et al., 1985b).

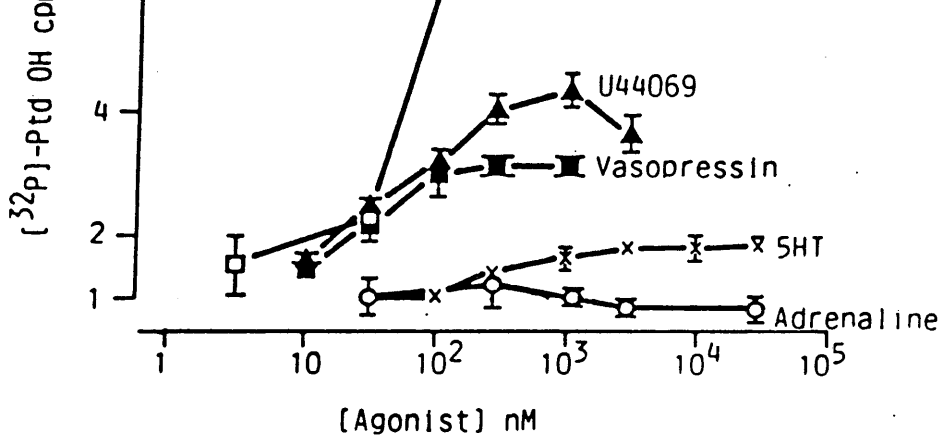

Figure 12. Agonist induced $[^{32}P]$ Ptd OH formation in human platelets.

Agonists, at the concentration indicated, were added to $[^{32}P]$ pre-labelled platelets. Levels of $[^{32}P]$ Ptd OH were measured 30s after the addition of PAF, vasopressin, U44069 and adrenaline and 60s after addition of 5HT. Results are expressed as "fold stimulation" of the basal value (= 1) (Figure 5 in MacIntyre et al., 1985b).

the possibility exists that thrombin induced platelet activation is not solely as a consequence of this binding. As thrombin has proteolytic activity, the possibility of this contributing to its mode of action was investigated.

TxA_2 mimetics such as U44069 and EP171 are routinely used to investigate the effects and mechanisms of action of the natural agonist on platelets and other TxA_2 sensitive tissues. However, which agent most closely mimics the actions of TxA_2 remains to be determined. For example, it has been shown that U44069 acts as a partial agonist at the thromboxane receptor (TP type) on airway and vascular smooth muscle (Jones et al., 1982). Although widely used to probe the sequelae of platelet TP receptor occupancy, it remains to be proven whether U44069 also acts as a partial agonist in this system. Indeed, U44069 and TxA_2 reportedly differ in their effects on inhibition of platelet adenylate cyclase (Gorman et al., 1978; Best et al., 1979). Besides this inhibition of adenylate cyclase, platelet TP receptors are coupled to inositol phospholipid hydrolysis and elevation of $\left[Ca^{2+}\right]_i$ (Pollock et al., 1984). Therefore the possibility that U44069 acts as a partial agonist at the platelet TP receptor was investigated.

3. Desensitisation

Platelet activation, which is a reversible process, exhibits the phenomenon of desensitisation. However, the underlying biochemical mechanism has not been widely investigated. The possibility exists that inhibitory second messengers may play a role in terminating, limiting or desensitising platelet

reactivity. Specifically the effects of several putative "negative feedback regulators" of agonist induced inositol phospholipid hydrolysis and elevation of $[Ca^{2+}]_i$ were examined.

3.1 Adenylate cyclase stimulants

Agents such as PGI_2, PGE_1 and PGD_2 which stimulate adenylate cyclase, have been shown to be inhibitors of platelet activation (Feinstein et al., 1983). Therefore, if an endogenous mediator of desensitisation exists, then it is possible that it may be cAMP. However there is no evidence that cAMP levels are elevated following exposure of platelets to stimulatory agonists or in desensitised platelets, although changes may occur in cAMP compartments in the absence of a demonstrable elevation in the total cellular cAMP. There is evidence that adenylate cyclase activation has a pronounced inhibitory action on platelet phosphoinositide hydrolysis (Lapetina, 1984) and $[Ca^{2+}]_i$, and this was further investigated in the present study. In addition this study would help clarify possible interactions between platelet second messengers.

3.2 Guanylate cyclase stimulants

The potent guanylate cyclase stimulant NaNP has been shown to inhibit agonist induced platelet activation (Glusa et al., 1974; Haslam et al., 1980), phosphoinositide hydrolysis (Takai et al., 1981b) and elevated $[Ca^{2+}]_i$ (Kawahara et al., 1984). In addition elevated intracellular cGMP levels have been reported in response to several agonists (White et al., 1973) and so cGMP is a prime candidate as a putative endogenous

regulator of platelet reactivity.

3.3 Protein kinase C stimulants

The phorbol esters have been shown to stimulate platelet reactivity (Zucker et al., 1974; White et al., 1974), presumably by mimicking endogenous DAG and stimulating protein kinase C (Castagna et al., 1982) and hence bypassing receptor mediated phosphoinositide hydrolysis. The phorbol esters however have been shown to influence agonist induced elevation of $[Ca^{2+}]_i$ in neutrophils (Serhan et al., 1983; Gennaro et al., 1984). Consequently, the effect of these agents on inositol phospholipid and $[Ca^{2+}]_i$ in resting and stimulated platelets were investigated.

4. Potentiation

The actions of adrenaline and ADP to potentiate the effects of other agonists are well documented. However the mechanism(s) of this potentiation is/are unknown. The possibility that this occurs by activity at the level of phosphoinositide hydrolysis was examined.

5. New techniques

The limitations of $[^{32}P]\,P_i$ prelabelled platelets to monitor inositol phospholipid hydrolysis have been discussed earlier. This study was involved with the development of two techniques which would help to confirm findings from such labelling studies.

5.1 Inorganic phosphorous assays

Inorganic phosphorous assays have been widely used to monitor phosphoinositide hydrolysis. It was hoped to adapt such an assay to be used to monitor the effects of a variety of agonists on PIP_2, PIP, Ptd Ins and Ptd OH levels in the human platelet.

5.2 Platelet permeabilisation

The use of $[^3H]$ inositol labelling and pertussis and cholera toxins pretreatment in human platelets have been precluded due to the inability of each of these compounds to cross the cellular membrane. The possibility of the permeabilisation technique, adapted for the incorporation of aequorin into the platelet (Johnson et al., 1985), being used for increasing the uptake of inositol and the toxins, was examined.

MATERIALS AND METHODS

CHAPTER VI: MATERIALS AND METHODS

1. Preparation of platelets

1.1 Preparation of platelet rich plasma

Blood was taken from the antecubital vein of apparently healthy human volunteers who denied taking any drugs known to affect platelet function. The blood was collected in disposable plastic centrifuge tubes containing 10% (v/v) sodium citrate (3.8% in GDW) as an anti-coagulant and centrifuged at 800g for 5 minutes. The supernatant (platelet rich plasma, PRP) was aspirated into other disposable centrifuge tubes. The remainder, red blood cells and leucocytes, was discarded. All experiments were completed within four hours of the removal of blood to maximise platelet viability (MacIntyre & Pollock, 1983).

1.2 Platelet washing procedures

1.2(a) Method I:

The platelets were pelleted by centrifugation of PRP at 800g for 10 minutes in the presence of an adenylate cyclase stimulant, either prostacyclin (100 ng/ml) or a mixture of PGE_1/ 6-keto-PGE_1 (100 ng/ml), to prevent platelet activation (Moncada et al., 1982). The supernatant was aspirated and discarded and the pellet was resuspended in the relevant buffer (MacIntyre & Pollock, 1983).

1.2(b) Method II:

A column of Sepharose 2B was equilibrated with the relevant buffer. The PRP was then washed through with additional buffer separating the platelets from the plasma (Pollock et al., 1984).

1.3 Buffers

1.3(a) Buffer I-Phosphate-free, calcium-free Hepes buffer:

150 mM-sodium chloride; 5 mM-Hepes

4 mM-potassium chloride; 10 mM-dextrose

1 mM-magnesium chloride; 0.3%-bovine serum albumin

(The pH was adjusted to 7.4).

1.3(b) Buffer II-Hepes-buffered Tyrodes' solution:

129 mM-sodium chloride; 0.35%-bovine serum albumin

10.9 mM-trisodium citrate; 2.4 mM-calcium chloride

8.9 mM-sodium bicarbonate; 0.56 mM-dextrose

2.8 mM-potassium chloride; 5 mM-Hepes

0.8 mM-potassium phosphate; 0.84 mM-magnesium chloride

(The pH was adjusted to 7.4).

1.3(c) Buffer III-Hepes buffered solution:

129 mM-sodium chloride; 1 mM-calcium chloride

8.9 mM-sodium bicarbonate; 10 mM-Hepes

2.8 mM-potassium chloride; 5.6 mM-dextrose

0.8 mM-potassium phosphate; 0.8 mM-magnesium chloride

(The pH was adjusted to 7.4).

1.3(d) Buffer IV-Permeabilisation buffer:

150 mM-sodium chloride; 5 mM-ATP

5 mM-Hepes; 1 μM-PGE_1

(The pH was adjusted to 7.4).

2. Labelling procedures

2.1 [^{3}H] -arachidonic acid

[^{3}H] -arachidonic acid (2 μCi/ml) was dried down at 25°C in a disposable centrifuge tube under a stream of oxygen free nitrogen. The PRP was then added and incubated at 37°C for 60 minutes. The PRP was washed by Method I and the platelets were resuspended in phosphate-free, calcium-free Hepes buffer (buffer: PRP; 1:2).

2.2 [^{3}H] -inositol

The relative impermeability of the platelet plasma membrane to inositol makes incubation of the platelets with [^{3}H] -inositol alone an unviable proposition. The platelets therefore have to be permeabilised. The only method which would permeabilise and reseal the plasma membrane was that of Salzman and colleagues (Johnson et al., 1985).

PRP (15-18 ml) was washed by Method I and the platelets resuspended in 1 ml of Hepes buffered solution (4°C). The platelets were immediately centrifuged (4000g; 20 seconds) and the supernatant aspirated and discarded. The platelets were resuspended in 1 ml of the permeabilisation buffer containing magnesium chloride (2 mM), EGTA (10 mM) and [^{3}H] -inositol (20 μCi) and incubated at 4°C for 60 minutes. The platelets were re-centrifuged (4000g; 20 seconds) and the supernatant aspirated and discarded. The platelets were resuspended in 1 ml of the permeabilisation buffer containing magnesium chloride (10 mM) and EGTA (0.1 mM) and incubated at 4°C for 60 minutes.

Three aliquots (1 µl) of permeabilisation buffer containing magnesium chloride (10 mM), EGTA (0.1 mM) and calcium chloride (100 mM) were added 5 minutes apart and the platelets were gently re-warmed to 22°C. The platelets were then re-washed by Method II and resuspended in Hepes buffered solution containing calcium chloride (1 mM) (Buffer:PRP; 2:3). These platelets were then tested in a platelet aggregometer for functional viability and were found to respond to high concentrations of agonists.

In order to assess the success of the permeabilisation procedure samples of these platelets were added to scintillation fluid and counted in a Liquid Scintillation Counter. It was found that there was no significant difference in the ^{3}H levels in these samples when compared to parallel platelets which had been prepared as above except that the incubations at 4°C were carried out in Hepes buffered solution containing EGTA (100mM) and PGE_1 (100 µg/ml). Therefore it appears either that the platelets have not undergone the permeabilisation procedure or the intracellular inositol levels were too high for the $[^{3}H]$-inositol to enter down its concentration gradient.

2.3 $[^{32}P]$-orthophosphate

2.3(a) Prelabelling for Ptd Ins and Ptd OH:

PRP was washed by Method I, the platelets resuspended in phosphate-free, calcium-free Hepes buffer (PRP:buffer; 5:1) and 25 µCi/ml $[^{32}P]$-orthophosphate was added. The cells were then incubated at 37°C for 90 minutes. The platelets were

re-washed by Method I, which removed the excess label, resuspended in phosphate-free, calcium-free Hepes buffer (PRP:buffer; 5:2) and allowed to equilibrate for 5 minutes prior to the start of the experiment (MacIntyre & Pollock, 1983). In selected experiments the cyclo-oxygenase inhibitor flurbiprofen (10 μM) was added in order to abolish the effects of thromboxane release. Under these conditions the labelling of Ptd OH had reached equilibrium with $[^{32}P]$-ATP and therefore changes in Ptd OH label reflect changes in Ptd OH mass (Holmsen et al., 1984). In addition the platelets remained functionally viable as indicated by aggregation studies.

2.3(b) Pulse chase labelling for PIP and PIP_2:

PRP was washed by Method I, the platelets were resuspended in phosphate-free, calcium-free Hepes buffer (PRP:buffer; 5:1) and incubated at 37°C for 45 minutes in the presence of $[^{32}P]$-orthophosphate (25 μCi/ml). This procedure labelled all three inositol phospholipids and Ptd OH. In order to enhance the possibility of observing agonist-induced changes in PIP and PIP_2 it was necessary to prevent the specific activity of the phosphoinositides from increasing. This was achieved by rewashing the cells by Method I and resuspending in fresh buffer (PRP:buffer; 5:2) containing KH_2PO_4 (100 μM) and incubating at 37°C for a further 45 minutes. This had the effect of chasing the label from the ATP pool with a resulting decrease in the specific activity of Ptd Ins (Pollock, 1984).

3. Experimental procedures

3.1 Measurement of [^{32}P]-Ptd OH and [^{32}P]-Ptd Ins

3.1(a) Experimental:

Aliquots (0.4 ml) of prelabelled platelets were dispensed into 3 ml plastic tubes in a 37°C water bath and allowed to stand for 5 minutes. Reactions were initiated by the addition of the drug(s) (or vehicle) in a 4 µl volume. At the appropriate time after addition, the reaction was terminated by transferring the entire sample to a glass test-tube containing 2 ml of chloroform:methanol:10N hydrochloric acid (25:50:4; v:v:v) and vortexing thoroughly. Each experiment contained a maximum of 24 samples, including vehicle controls at the start and end of the experiment, and protocols were designed to minimise the duration of each experiment.

3.1(b) Extraction of phospholipids:

The phospholipids were extracted by Method B of Lloyd *et al.* (1972). This involved the addition of 0.625 ml of chloroform and 0.625 ml of GDW and vortexing thoroughly. The samples were centrifuged (1000g, 3 minutes) which caused a partition into two distinct phases and a protein interface. The lower, organic, phase was removed by Pasteur pipette to a drying bottle and the upper, aqueous, phase and protein interface were discarded. The phospholipids were dried down at 45°C under a constant stream of oxygen free nitrogen. The drying bottle was then sealed and stored at -20°C for a maximum of 3 days.

3.1(c) Separation of phospholipids:

The phospholipids were separated by two dimensional thin layer chromatography (t.l.c.) (Yavin & Zutra, 1977). Silica-gel t.l.c. plates (10 cm x 10 cm) were marked 2 cm x 2 cm from the lower left edge and 5 µg of carrier Ptd OH was spotted. The phospholipids were redissolved in 75 µl of chloroform: methanol (9:1; v:v) and were then spotted onto the t.l.c. plate. The plates were then run in the first dimension in a solvent system of chloroform:methanol:40% methylamine (26:12:3; v:v:v). The plates were removed when the solvent front had reached 1-2 cm from the top of the plate. The basic nature of this system meant that the acidic phospholipids, such as Ptd Ins and Ptd OH, were retarded. The t.l.c. plates were dried under a stream of warm air, subjected to hydrochloric acid fumes to neutralise the methylamine and dried initially under warm air and then cool air. The plates were then subjected to an acidic wash in the second dimension in a solvent system of diethyl ether: acetic acid (19:1) and dried under a stream of cool air. The lipids were separated in the second dimension in a solvent system of chloroform:acetone:methanol:acetic acid: GDW (10:4:2:3:1 The solvent front in this dimension was allowed to run no further than that of the acidic wash. All solvents were renewed after each group of 24 plates and allowed 60 minutes to equilibrate. The plates were placed in iodine vapour which allowed the identification of Ptd Ins, Ptd OH, Ptd Cho, Ptd Eth, Ptd Ser, lyso-Ptd Eth and sphingomyelin. However under the labelling conditions employed only Ptd Ins, Ptd OH and Ptd Cho became

labelled to a significant extent (Figure 13). The spots corresponding to Ptd Ins and Ptd OH were scraped into a scintillation vial and counted in a Liquid Scintillation Counter.

3.2 Measurement of $[^{32}P]$-PIP and $[^{32}P]$-PIP_2

3.2(a) Experimental:

Aliquots (0.4 ml) of pulse chase labelled platelets were dispensed into 3 ml plastic tubes and allowed to stand at 37°C for 5 minutes prior to use. Reactions were initiated by the addition of drug(s) (or vehicle) in a volume of 4 µl. At the appropriate time the reactions were terminated by transferring the entire sample to a glass test tube containing 1.2 ml of chloroform:methanol (1:2) and vortexing thoroughly. Each experiment contained a maximum of 24 samples, including vehicle controls at the beginning and end of the experiment, and were designed to be of minimum duration.

3.2(b) Extraction of phospholipids:

The phospholipids were extracted by the addition of 2.4N hydrochloric acid (0.4 ml) and chloroform (0.4 ml) and then vortexing. The sample was partitioned into two phases by centrifugation (1000g; 3 minutes) and the lower, organic phase was removed to another glass test tube by Pasteur pipette and stored at 4°C. The aqueous phase was rewashed by mixing with chloroform (0.8 ml) and centrifugation (1000g; 3 minutes) to separate phases. The upper, aqueous phase was removed by Pasteur pipette and discarded and the organic phases combined.

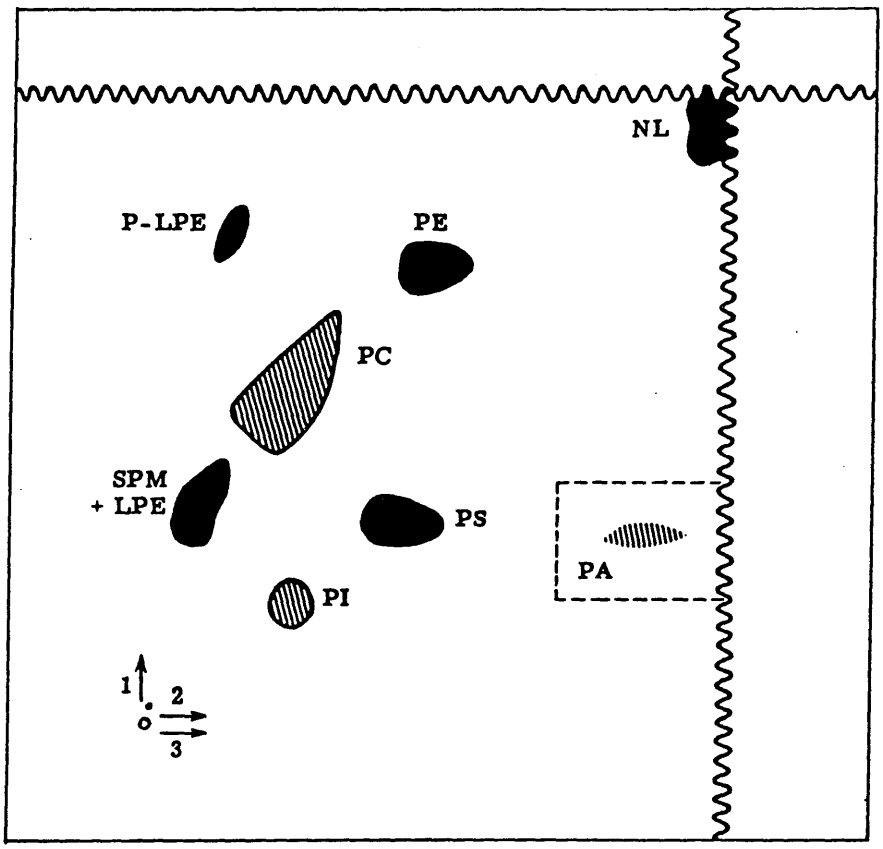

Figure 13. Diagramatic representation of phospholipid separation by method of Yavin and Zutra (1977).

Phospholipids identified by iodine staining are indicated and the hatched spots represent those phospholipids which are significantly labelled following preincubation with $[^{32}P]$ P_i.
PI - phosphatidylinositol; PS - phosphatidylserine; PA-phosphatidic acid; LPE-lyso phosphatidylethanolamine; SPM-sphyngomyelin; PC-phosphatidylcholine; PE-phosphatidylethanolamine; P-LPE-plasmalogen lyso phosphatidylethanolamine; NL-neutral lipids.

The organic phases were washed by the addition of 1.6 ml of methanol : 1N hydrochloric acid (1:1), centrifuged (1000g; 3 minutes) and the lower, organic phase removed, by Pasteur pipette, to a drying bottle. The phospholipids were dried down at 45°C under a stream of oxygen free nitrogen, the bottle sealed and stored at -20°C for a maximum of 3 days (Schacht, 1981).

3.2(c) Separation of phospholipids:

High performance t.l.c. plates (10 cm x 20 cm) were impregnated with potassium oxalate (1% in methanol:GDW (2:3)) and activated by heating at 110°C for at least 10 minutes. The phospholipids were resuspended in 75 µl of chloroform:methanol: GDW (75:25:2) and spotted in 1.5 cm bands 2 cm from the edge of the plate. The plates were then placed in a solvent system of chloroform:acetone:methanol:acetic acid:GDW (40:15:13:12:8) and allowed to run until the solvent front reached the top edge of the plate (Jolles et al., 1981). Fresh solvents were used for each experiment and allowed 60 minutes to equilibrate. The plates were dried and then autoradiographed on X-Omat S X-ray film (Kodak). This allowed the identification of PIP_2, PIP, Ptd Ins and Ptd OH. Spots corresponding to PIP_2 and PIP were scraped into a scintillation vial and counted in a Liquid Scintillation Counter.

3.3 Measurement of $[^3H]$ -arachidonate release

Platelets prelabelled with $[^3H]$ -arachidonic acid were dispensed (0.2 ml) into disposable 1.5 ml plastic tubes at 37°C. Reactions were initiated by the addition of drug(s) (or vehicle), in a volume of 4 µl, and terminated by the addition of ice cold EDTA/saline and removing the entire tube onto ice. The samples were then centrifuged (2000g; 4 minutes; 4°C) to pellet the platelets and an aliquot of supernatant was counted in a Liquid Scintillation Counter.

3.4 Direct measurement of phospholipid mass

The measurement of the cellular content of phospholipid by the use of radiolabel have several deficiencies. Therefore the direct measurement of the mass of lipid present has been used in some studies. These studies have utilised the many assays which have been developed to estimate the content of inorganic phosphorous and several have been discussed earlier.

The phospholipids were separated by t.l.c. and the spots scraped into Pasteur pipettes which had been plugged with glass wool and washed thoroughly with chromic acid. The phospholipids were eluted from the silica by passing 5 ml of chloroform: methanol:10N hydrochloric acid (25:50:4) through the pipette. The eluant was collected and dried down (45°C) under a stream of oxygen free nitrogen. The phospholipids were digested to release the inorganic phosphorous by adding 0.1 ml of perchloric acid and heating (170-180°C; 60 minutes), taking care to avoid loss of the perchloric acid fumes. The samples were then cooled. On each occasion a standard curve was

constructed by using either potassium dihydrogen phosphate, or a known amount of phospholipid, usually Ptd Cho.

3.4(a) Method of Bartlett (1959) (modified by Christie (1982)):

A solution of ammonium molybdate (2.4 ml) was added, followed by a reducing agent (2.4 ml). The samples were thoroughly mixed, placed in a boiling water bath (10 minutes) and the extinction at 830 nm read in a standard Cecil spectrophotometer. The ammonium molybdate solution consisted of 4.4g of ammonium molybdate in 500 ml of GDW, 14 ml of concentrated sulphuric acid was added and the solution was made up to 1 litre with GDW. The reducing agent was sodium(meta)bisulphite (2.5g), sodium sulphite (0.5g) and 1 amino 2 naphol 4 sulphonic acid (0.042 g) dissolved in 250 ml of GDW and allowed to stand in the dark for several hours. The solution was filtered and stored at $4^{o}C$.

A typical standard curve using this method is shown in Figure 14. The inorganic phosphorous content of Ptd Ins was estimated using this technique. The mean content ($\pm$ SEM) was 12.5 ($\pm$ 4.4) nmoles phosphorous per sample, which converted to 57.9 ($\pm$ 20.4) nmoles per 5 x 10^9 platelets. This, although the error involved was large, is consistent with the published values of 61.55 nmoles per 5 x 10^9 platelets (Broekman et al., 1980). Thrombin (1 U/ml) caused a decrease in the amount of phosphorous to 33.9 ($\pm$ 4.4) nmoles of phosphorous per 5 x 10^9 platelets, however this effect was at the limit of sensitivity of this assay. It also proved impossible to quantify the phosphorous levels in Ptd OH and for these reasons this technique was discontinued.

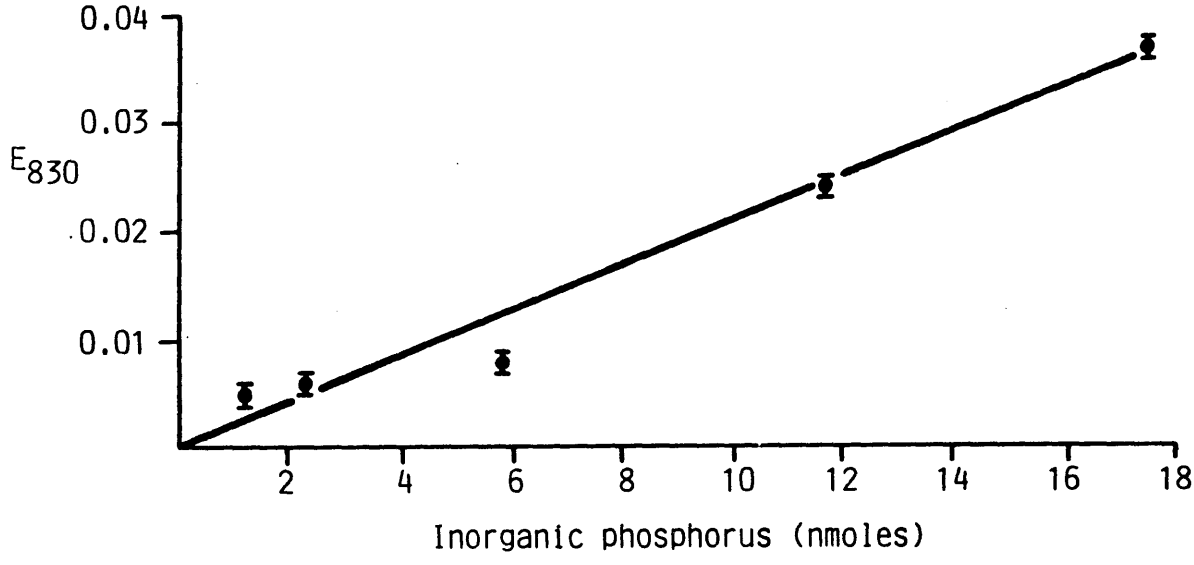

Figure 14. Measurement of inorganic phosphorous by the method of Bartlett (1959) as modified by Christie (1982).

Typical standard curve using the procedure outlined in Materials and Methods (3.4a).

3.4(b) Method of Bowyer and King (1977):

The samples were vortexed with 0.5 ml of GDW and centrifuged (1000g; 5 minutes). A standard aliquot (0.5 ml) was removed, thoroughly shaken with 1.5 ml of the "colour reagent" and the extinction read at 660 nm. The "colour reagent" was a mixture of 1 volume of ammonium molybdate (4.2% in 5N hydrochloric acid) and 3 volumes of malachite green (0.2% in GDW). The mixture is allowed to stand for 30 minutes and filtered before use.

A typical standard curve using this procedure is shown in Figure 15. It can be readily seen that it was impossible to obtain the sensitivity to measure the mass of phosphorous in the phospholipids.

3.4(c) Method of Rouser et al. (1969):

The following reagent were added rapidly and in order to the samples which were thoroughly vortexed after each addition: (i) 0.92 ml of GDW; (ii) 0.4 ml of ammonium molybdate (1.25% in GDW); (iii) 0.4 ml of ascorbic acid (5% in GDW). The samples were incubated in a boiling water bath for 5 minutes and the extinction read at 797 nm.

A typical standard curve is shown in Figure 16. This technique indicated a phosphorous content of Ptd Ins of only 9.7 nmoles per 5×10^9 platelets and the effects of thrombin and the basal levels of Ptd OH were undetectable.

3.4(d) Conclusions:

Each of the above assays were relatively insensitive to the measurement of basal Ptd OH levels and the effects of an agonist on both Ptd Ins and Ptd OH. An additional complication

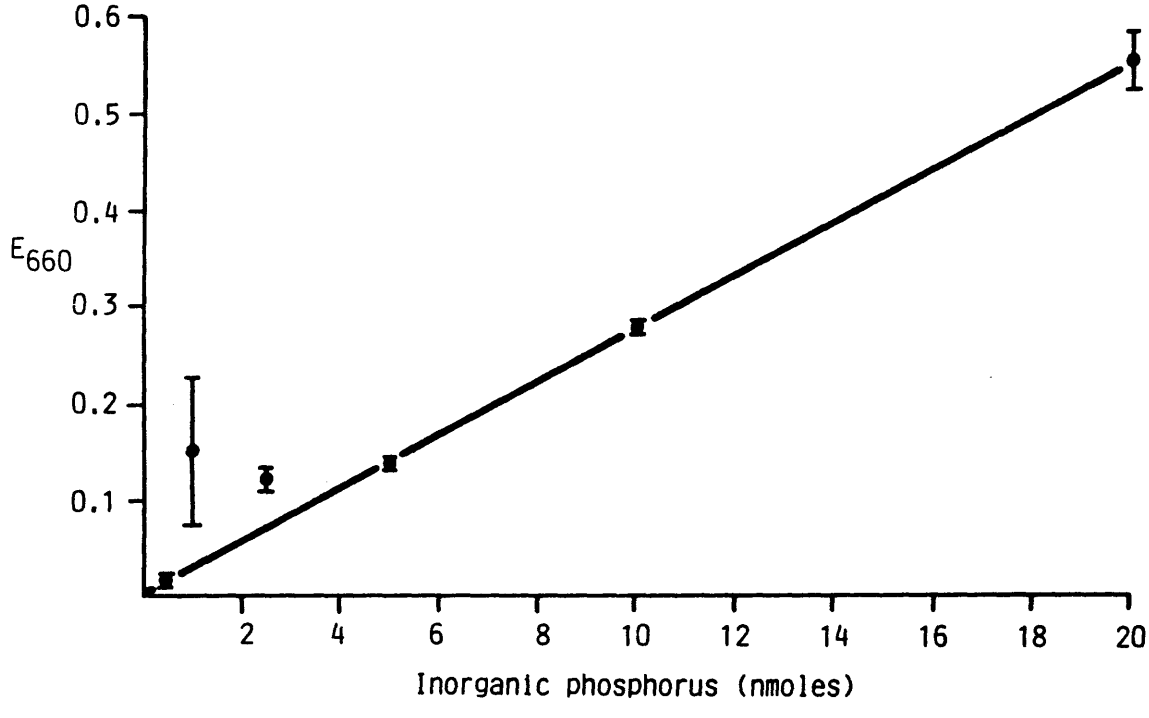

Figure 15. Measurement of inorganic phosphorous by the method of Bowyer and King (1977).

Typical standard curve using the procedure outlined in Materials and Methods (3.4b).

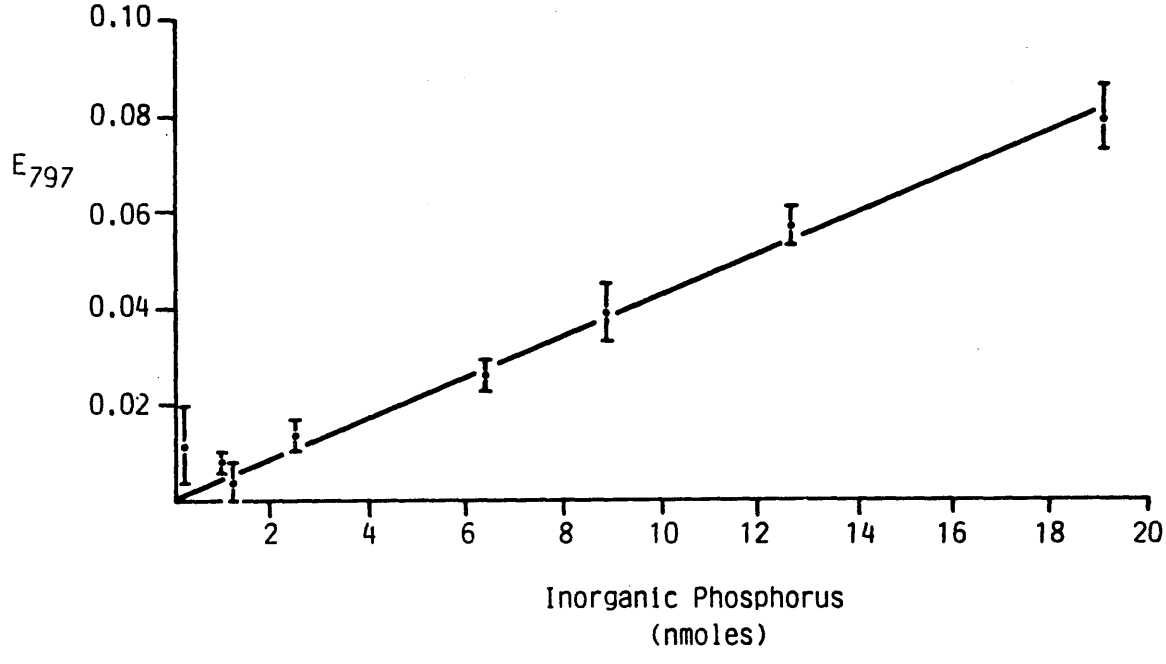

Figure 16. Measurement of inorganic phosphorous by the method of Rouser et al. (1969).

Typical standard curve using the procedure outlined in Materials and Methods (3.4c).

encountered during the measurement of Ptd OH was the fact that there is so little Ptd OH present in the cell that it is not readily seen on a t.l.c. plate without the use of carrier. The use of such a carrier in the measurement of the mass of Ptd OH was impossible as it would affect the resultant calculation and so the position of Ptd OH on the plate had to be estimated using R_f values. These problems of insensitivity could, of course, be overcome by increasing the platelet count per sample, which could be achieved by either increasing the amount of blood drawn from a single donor or by pooling blood. The former proposition was ruled out due to the fact that there was only a limited supply of regular donors and large volumes of blood could not be donated on a routine basis. The pooling of blood was excluded as it too would necessitate the use of large amounts of blood if quantitative observations were to be made. Therefore these experiments were discontinued on the grounds that they were time consuming, insensitive, expensive in terms of blood and alternative approaches were available.

3.5 Measurement of intracellular calcium concentrations

Intracellular calcium levels were monitored by using the fluorescent calcium dye Quin 2 (Tsein *et al.*, 1982; Pollock *et al.*, 1984). PRP was incubated with Quin 2 acetoxymethyl ester (10 μM; 37°C; 30 minutes) after which the platelets were washed by Method II using the Hepes-buffered Tyrodes' solution. The Quin 2 content of the platelets using this technique was 1 mmole

per litre of cell water. The external free calcium concentration was adjusted to 1 mM immediately before use by the addition of calcium chloride. Platelet samples (2 ml; approximately 5×10^7 platelets per ml) were placed in 1 cm square quartz curvettes at 37°C and the fluorescence monitored (Perkin-Elmer LS3 Fluorescence Spectrometer) with standard monochromator settings of 339 nm (excitation) and 500 nm (emission). During the course of each experiment at least one sample was treated with digitonin (50 μM) to lyse the platelets thereby releasing all cellular calcium. This indicated the maximum calcium levels present. This sample was then treated with EGTA (20 mM) which bound all calcium present and so gave a theoretical minimum calcium level. The intracellular calcium concentration was obtained using the following equation:-

$$\frac{F - F_{min}}{F_{max} - F} \times 115$$

where

F = measured fluorescence

F_{min} = measured fluorescence in presence of digitonin and EGTA

F_{max} = measured fluorescence in presence of digitonin

A typical representative trace of the fluorescence record obtained in Quin 2 labelled platelets is shown in Figure 17.

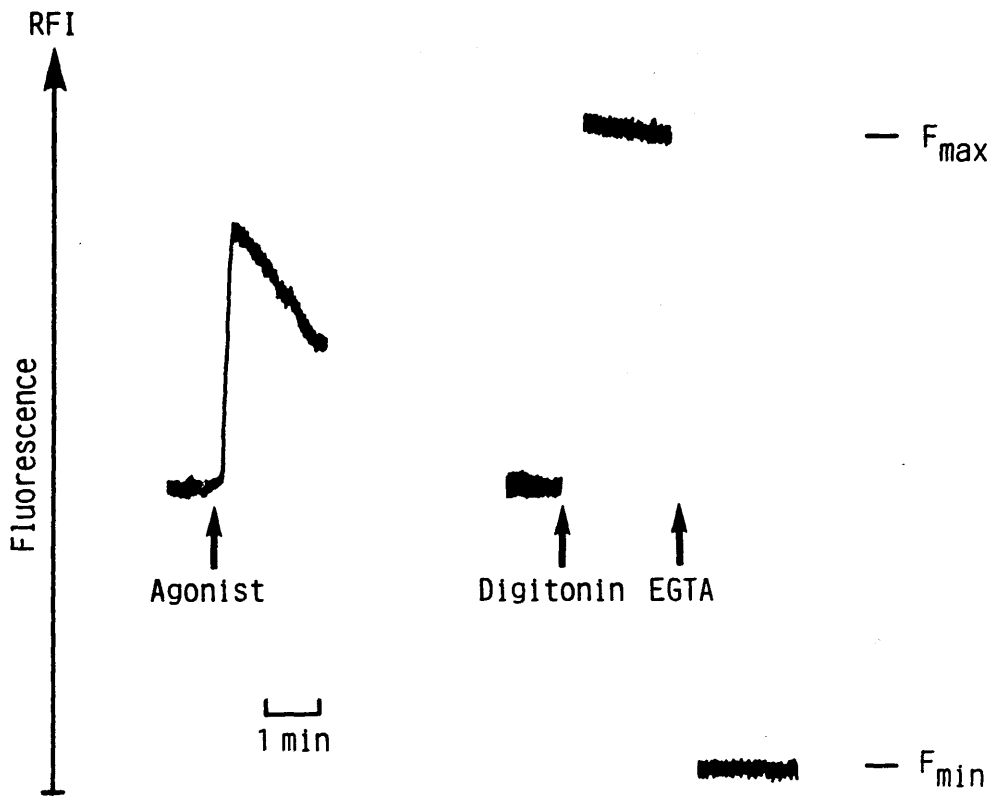

Figure 17. Diagramatic representation of fluorescence record in Quin 2 labelled platelets.

Typical representative trace of fluorescence in Quin 2 labelled platelets. Agonist addition elicits an increase in fluorescence which can be readily calibrated for $[Ca^{2+}]_i$. F_{max} (theoretical maximum $[Ca^{2+}]_i$) obtained by the addition of digitonin. F_{min} (theoretical minimum $[Ca^{2+}]_i$) was obtained by subsequent addition of EGTA.

3.6 Measurement of platelet functions

3.6(a) Aggregation:

Platelet aggregation was measured using platelets washed by Method I and resuspended in Phosphate free, calcium free Hepes buffer. A platelet sample (0.6 ml) was placed in a standard Malin aggregometer. The agonist (6 µl) was added and the resultant aggregation compared to buffer alone (0.6 ml).

3.6(b) ATP release:

ATP release, an index of dense granular secretion, was measured directly by use of Lucifern-luciferase luminescence in platelets washed by Method I and resuspended in Phosphate free, calcium free Hepes buffer. Lucifern-Luciferase (15 µl) was added to a platelet sample (0.5 ml) in a Chronolog Corp. luminescence aggregometer, the agonist was added and aggregation and ATP secretion were measured simultaneously.

3.7 Quantification of radioactivity

All radioactive samples to be analysed were added to 5-10 ml of either scintillator-299 (Packard) or ecoscint (National Diagnostics) in polythene vials and counted (5 minutes) for their radioactive content in a liquid scintillation counter (Packard Tri Carb).

4. Calculation of results

Simple statistical analysis was carried out on results obtained from the liquid scintillation counter to give mean, standard deviation (SD) and standard error of mean (SEM). Vehicle controls were carried out at the beginning and the end of each experiment and the test values were expressed as a

percentage of these controls. Statistical significance was assessed using Student's t-test; only values of $p < 0.05$ were taken to indicate statistical significance and the level of significance is indicated at each experiment.

5. Materials

5.1 Drugs

Saline (0.9% in GDW) was used to dissolve, dilute and used as vehicle control for the following drugs:-
Bovine thrombin (Sigma), 8-arginine vasopressin (Sigma), adrenaline tartrate (Koch-Light Laboratories (U.K.), adenosine 5'-diphosphate (ADP, Sigma), sodium nitroprusside (NaNP, Hopkins & Williams), 8-bromoadenosine 3'5'cyclic monophosphate (8-bromo cAMP, Sigma), 8-bromoguanosine 3'5' cyclic monophosphoric acid (8-bromo cGMP, Sigma), bovine trypsin (Sigma), leupeptin (Sigma), flurbiprofen (Boots), EP 171 (Dr. R.L. Jones, University of Edinburgh) and the "PG mixture" of PGE_1/6-keto-PGE_1.

Phorbol 12 myristate 13 acetate (PMA, Sigma), 4-β-phorbol (Sigma), phorbol 12,13 dibutyrate (PDBu, Sigma) were dissolved in dimethyl sulphoxide (DMSO, Sigma) which was also used, at concentrations of less than 1%, as the vehicle control.

The stock (1.8 mM) platelet activating factor (PAF, Bachem, Babbendorf, Switzerland) was dissolved in iso-osmotic saline containing bovine serum albumin (0.25%). Subsequent dilutions of this stock were made up in saline which was also used as the vehicle control.

Prostacyclin (Dr J.E. Pike, Upjohn Co.) was dissolved (1 mg/ml stock), and dilutions made, in Tris buffer (50 mM in GDW, pH > 9).

PGD_2 (Dr J.E. Pike, Upjohn) was dissolved initially in ethanol and diluted to a 3.3 mM stock by addition of 9 volumes of sodium carbonate (3 mM in GDW). All subsequent dilutions of this stock were in saline.

5.2 Phospholipid standards

Phosphatidylinositol (Sigma) was dissolved in chloroform: methanol (2:1), phosphatidic acid (Sigma) in chloroform:methanol (1:1) and phosphatidylcholine (Sigma) in chloroform.

5.3 T.l.c. plates

Merck Silica Gel 60 high performance t.l.c. plates (10 cm x 20 cm) and Macherey-Nagel precoated t.l.c. plates SIL G-25 (20 cm x 20 cm) were purchased (MacFarlane Robson Ltd., Glasgow). The latter being cut into four smaller plates (10 cm x 10 cm) prior to use.

5.4 Radiochemicals

Carrier free $[^{32}P]$-orthophosphate was provided by the Regional Isotope Dispensary (Western Infirmary, Glasgow). 5,6,8,11,12,14,15- $[^{3}H]$ arachidonic acid and myo-$[2\text{-}^{3}H]$ inositol were purchased from Amersham International.

5.5 Others

Bovine serum albumin, Hepes, ethylene diamine tetra-acetic

acid (EDTA) and ethylene glycol bis (β-amino ethyl ether) tetra-acetic acid (EGTA) were purchased from Sigma, perchloric acid (70%) from BDH Ltd. (Poole), Quin 2 acetoxymethylester from Lancaster Synthesis (Morecambe) and all solvents and laboratory reagents were of analytical grade or better.

RESULTS

CHAPTER VII: RESULTS

1. Phosphoinositide hydrolysis

Although previous studies (Rittenhouse-Simmons, 1979; Broekman et al., 1980; Bell & Majerus, 1980; MacIntyre & Pollock, 1983; Agranoff et al., 1983; Pollock et al., 1984) have examined the effects of agonists on human platelet phosphoinositide metabolism, characterisation of these effects is by no means complete. Accordingly this section is concerned with investigating the effects of several agonists, including ADP, EP 171, trypsin and PMA, to ascertain whether phosphoinositide hydrolysis serves as the/a transduction process by which these agents generate second messenger molecules to influence human platelet function. In so doing, I examined the possibility that U44069 acts as a partial agonist at the platelet TxA_2 (TP) receptor. In addition I attempted to elucidate whether thrombin exerts its effects via both proteolytic and receptor-directed activities. In all cases the data, expressed as a percentage of the relevant control, are the mean $\pm$ SEM of triplicate determinations and in each case are typical of 2 to 4 similar experiments.

1.1 Effect of ADP on platelet phosphoinositide metabolism

If phosphoinositide hydrolysis plays a role in ADP induced platelet activation, it follows that those concentrations of the agonist which stimulate the cell should also elicit significant formation of $\left[^{32}P\right]$ Ptd OH. Preliminary studies established that platelets prepared under conditions of radio-labelling retained responsiveness (i.e. aggregation) to

ADP. Figure 18 depicts the concentration response relationship for ADP on platelet $[^{32}P]$ Ptd OH, the formation of which is the most sensitive index of inositol phospholipid metabolism in human platelets. In unstimulated platelets the levels of $[^{32}P]$ Ptd OH did not change significantly. When challenged with 0.1 - 100 μM ADP for 30s there was no significant formation of $[^{32}P]$ Ptd OH by platelets at any concentration of ADP.

It is possible that the time course of ADP stimulated phosphoinositide metabolism differs from that reported in response to other agonists (MacIntyre & Pollock, 1983; Pollock et al., 1984; Pollock & MacIntyre, 1986). Therefore platelets were challenged with a supramaximal (in terms of functional response) concentration of ADP (100 μM) for 10 - 300s and the effects on $[^{32}P]$ Ptd OH levels are shown in Figure 19. Once again there was no significant alteration in $[^{32}P]$ Ptd OH levels at any time.

The final index of ADP induced phosphoinositide metabolism monitored was the effect of the agonist on $[^{32}P]$ -PIP_2 and -PIP levels and the results are shown on Figure 20. The level of neither phospholipid was significantly affected by the presence of ADP (100 μM; 10 - 300s).

These observations indicate that ADP is unique amongst those agonists which have been reported to elevate $[Ca^{2+}]_i$ in human platelets (MacIntyre et al., 1985b) and are in contrast to reports in rabbit (Lloyd et al., 1972, 1973a; Vickers et al., 1982; Leung et al., 1983), rat (MacIntyre & MacMillan, 1985) and human (Vickers et al., 1983) platelets. In each of these

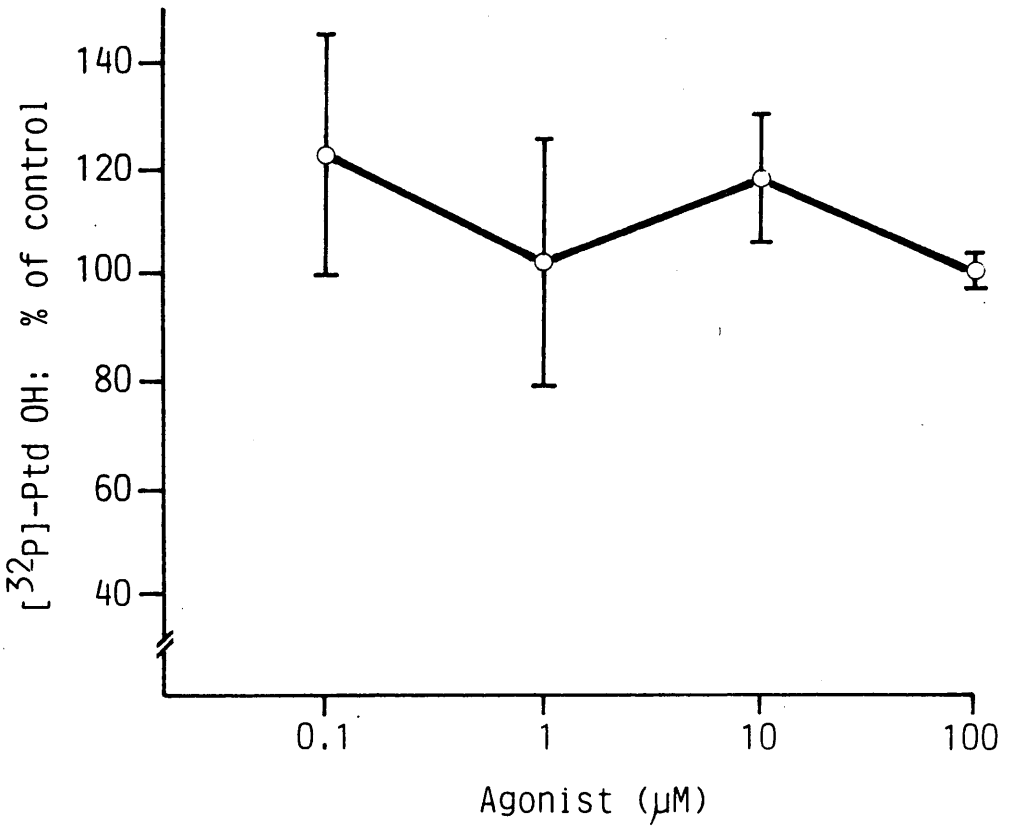

Figure 18. Concentration response curve for ADP-induced stimulation of $[^{32}P]$ Ptd OH formation

0.4 ml samples of $[^{32}P]$ P_i prelabelled platelets were exposed to ADP at the concentrations indicated. $[^{32}P]$ Ptd OH levels were measured 30s after agonist addition. The results are means ± SEM of triplicate determinations. The experiment shown is typical of 5 similar experiments.

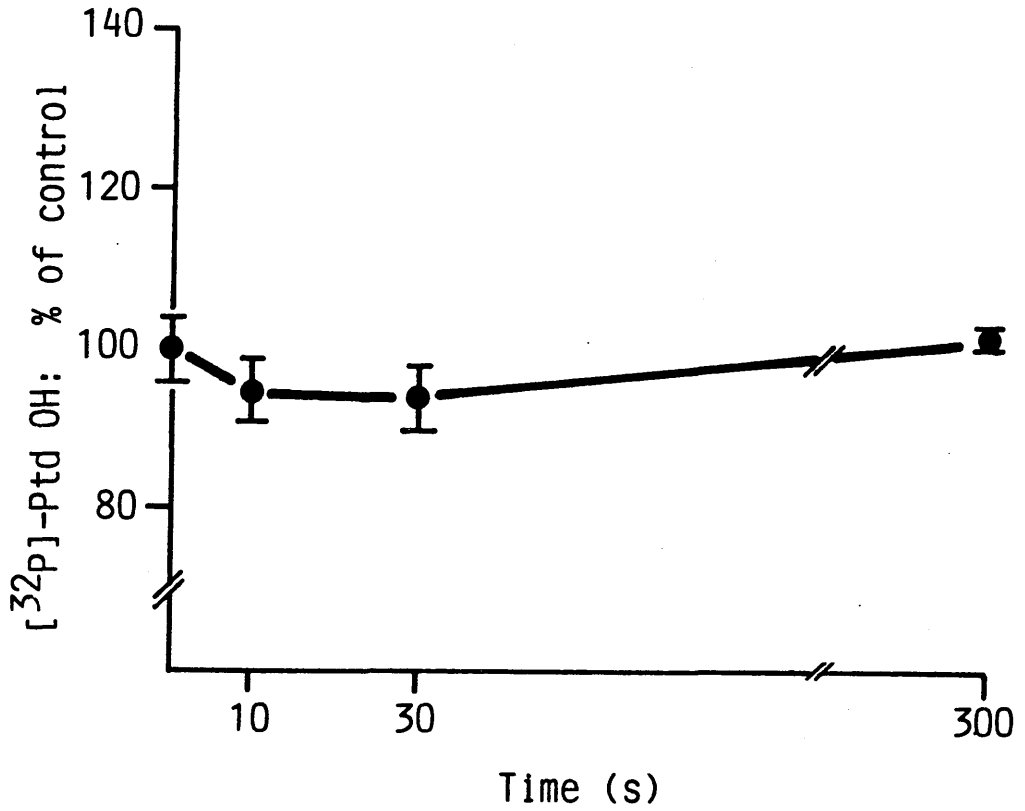

Figure 19. Time course of ADP-induced stimulation of $[^{32}P]$ Ptd OH formation.

0.4 ml samples of $[^{32}P]$ P_i prelabelled platelets were exposed to 100 μM ADP and the $[^{32}P]$ Ptd OH levels measured at the times indicated. The results are means ± SEM of triplicate determinations. The experiment shown is typical of 3 similar experiments.

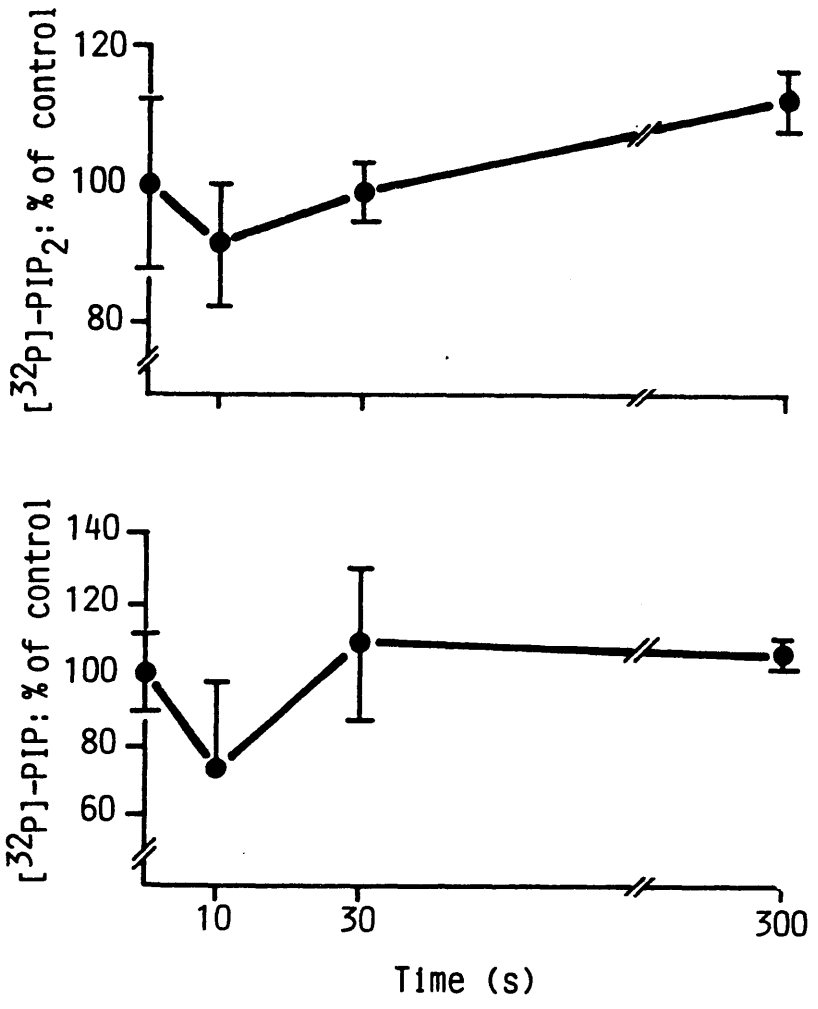

Figure 20. Time course of ADP-induced changes in levels of $[^{32}P]$ poly phosphoinositides.

0.4 ml samples of $[^{32}P]$ P_i pulse chase labelled platelets were exposed to 100 μM ADP and the levels of $[^{32}P]$ PIP_2 (upper trace) and $[^{32}P]$ PIP (lower trace) were measured at the times indicated. The results are means ± SEM of triplicate determinations. The experiment shown is typical of 3 similar experiments.

studies ADP was shown to stimulate phosphoinositide metabolism as monitored by Ptd OH formation and PIP_2 loss, measured in both mass and ^{32}P labelling studies. However in support of my observations, Fisher and colleagues (1985) subsequently confirmed that in human platelets, ADP does not affect $[^{32}P]$ -Ptd OH, -Ptd Ins, -PIP_2 and -PIP levels or the formation of $[^{3}H]$ inositol phosphates.

1.2 Effect of EP 171 on platelet phosphoinositide metabolism

It has been previously shown, using the stable analogue U44069 (9,11-epoxy methano PGH_2), that agonist occupancy of platelet TP receptors is coupled to phosphoinositide hydrolysis (Pollock et al., 1984). However studies on vascular tissue indicate that U44069 may be a partial agonist (Jones et al., 1982).

EP 171 is the most potent TxA_2 mimetic known: in platelets it induces shape change, aggregation, secretion and elevates $[Ca^{2+}]_i$ (Jones et al., 1985). I therefore compared the effects of EP 171, U44069 and a known partial agonist at the TP receptor, Et PGH_2 (9,11-ethano PGH_2), alone and in combination on phosphoinositide metabolism in order to assess whether U44069 is a full or partial agonist. EP 171 (1 nM - 625 nM; 120s) elicited a concentration dependent formation of $[^{32}P]$ Ptd OH (Figure 21). The maximal stimulation was 800 $\pm$ 20% of control with an EC_{50} of 9 $\pm$ 3 nM.

Using a high concentration of EP 171 the time course of changes in $[^{32}P]$ Ptd OH was also measured (Figure 22). EP 171 (625 nM) induced a rapid increase in $[^{32}P]$ Ptd OH which plateaued at 120 - 300s (602 ± 19% of control) and thereafter declined to 402 ± 19% at 600s. As this time course does not rapidly reach its maximum level it is consistent with the relatively slow onset of functional responses observed (Jones et al., 1985). In addition EP 171, like U44069, maintained elevated levels of $[^{32}P]$ Ptd OH until at least 600s following agonist stimulation (Pollock et al., 1984).

The effects of EP 171 (625 nM) on $[^{32}P]$ -PIP_2 and -PIP levels is shown in Figure 23. The agonist caused an initial decrease (by approximately 12 ± 1%) in the levels of PIP_2 followed by an increase (112 ± 4% at 600s). In contrast EP 171 caused no decrease in PIP but the subsequent increase was much larger than that of PIP_2 (315 ± 20% at 600s). These observations are further evidence that the effects of the platelet Tx receptor are mediated by phosphoinositide hydrolysis.

1.3 Comparison of the effects of U44069, Et PGH_2 and EP 171 alone and in combination on $[^{32}P]$ Ptd OH formation and ATP secretion

The possibility that U44069 was a partial agonist at the human platelet Tx receptor was investigated by examining the effects of U44069, EP 171 and the known partial agonist Et PGH_2 alone and in combination on platelet phosphoinositide metabolism. Figure 24 shows the concentration response relationships for

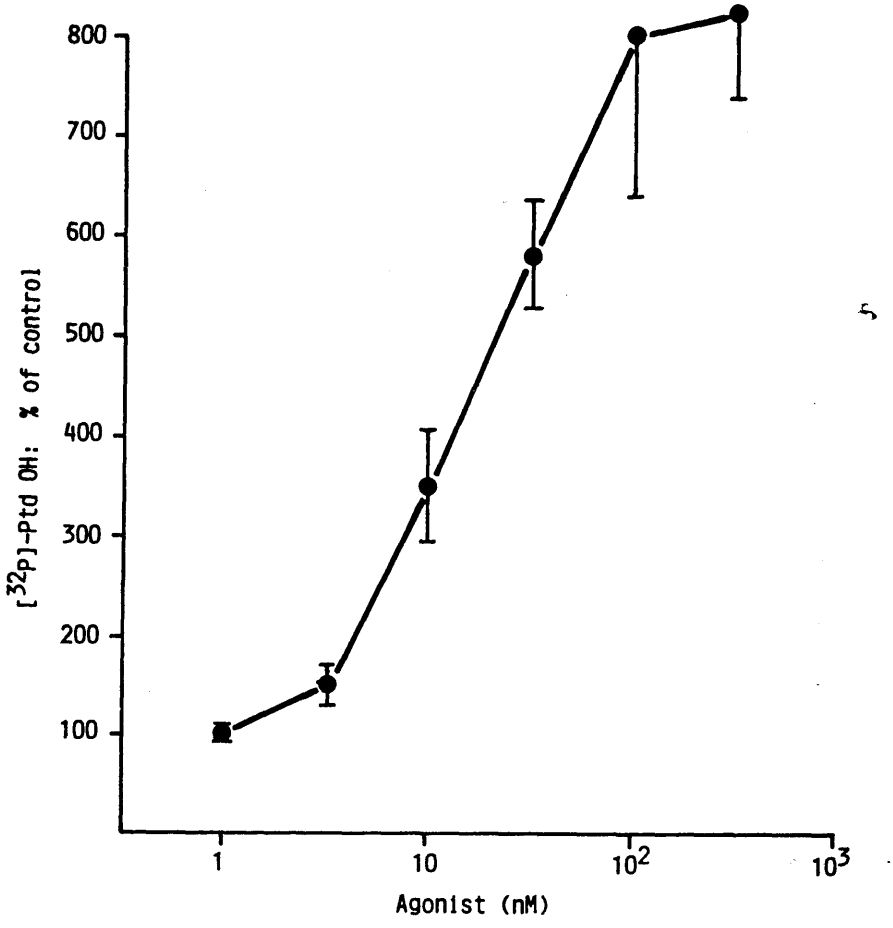

Figure 21. Concentration-response curve for EP 171-induced stimulation of $[^{32}P]$ Ptd OH formation.

0.4 ml samples of $[^{32}P]$ P_i prelabelled platelets were exposed to EP 171 at the concentrations indicated. $[^{32}P]$ Ptd OH levels were measured 120s after agonist addition. The results are means $\pm$ SEM of triplicate determinations. The experiment shown is typical of 3 similar experiments.

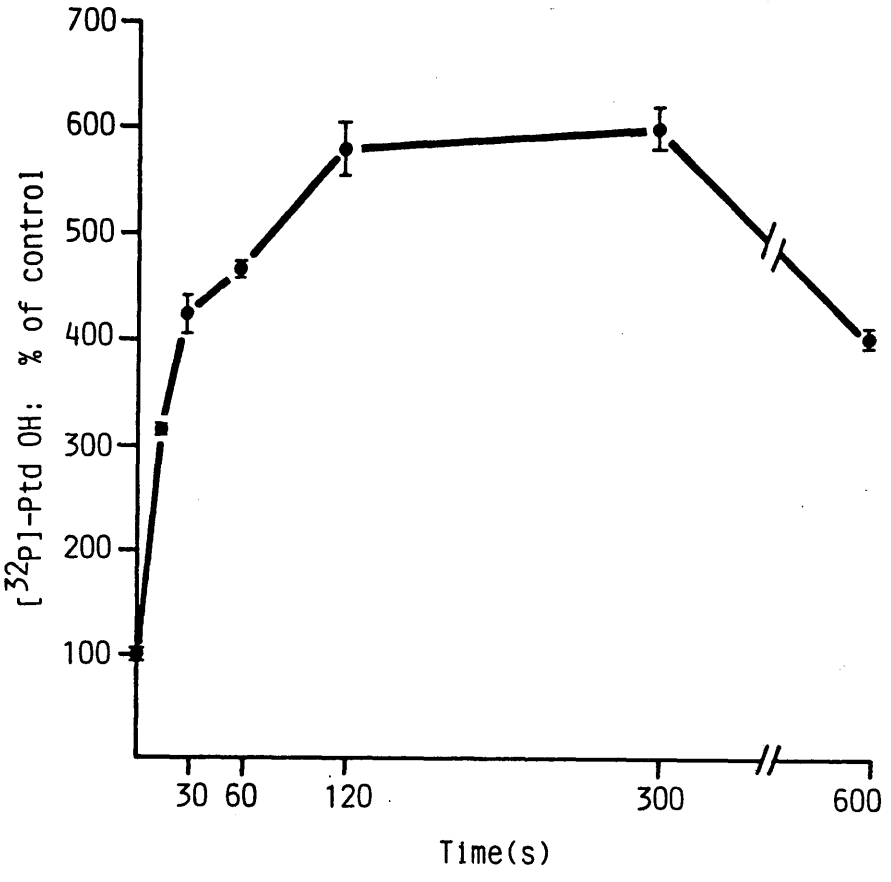

Figure 22. Time course of EP 171-induced stimulation of $[^{32}P]$ Ptd OH formation.

0.4 ml samples of $[^{32}P]$ P_i prelabelled platelets were exposed to 625 nM EP 171 and the levels of $[^{32}P]$ Ptd OH measured at the times indicated. The results are means ± SEM of triplicate determinations. The experiment shown is typical of 2 similar experiments.

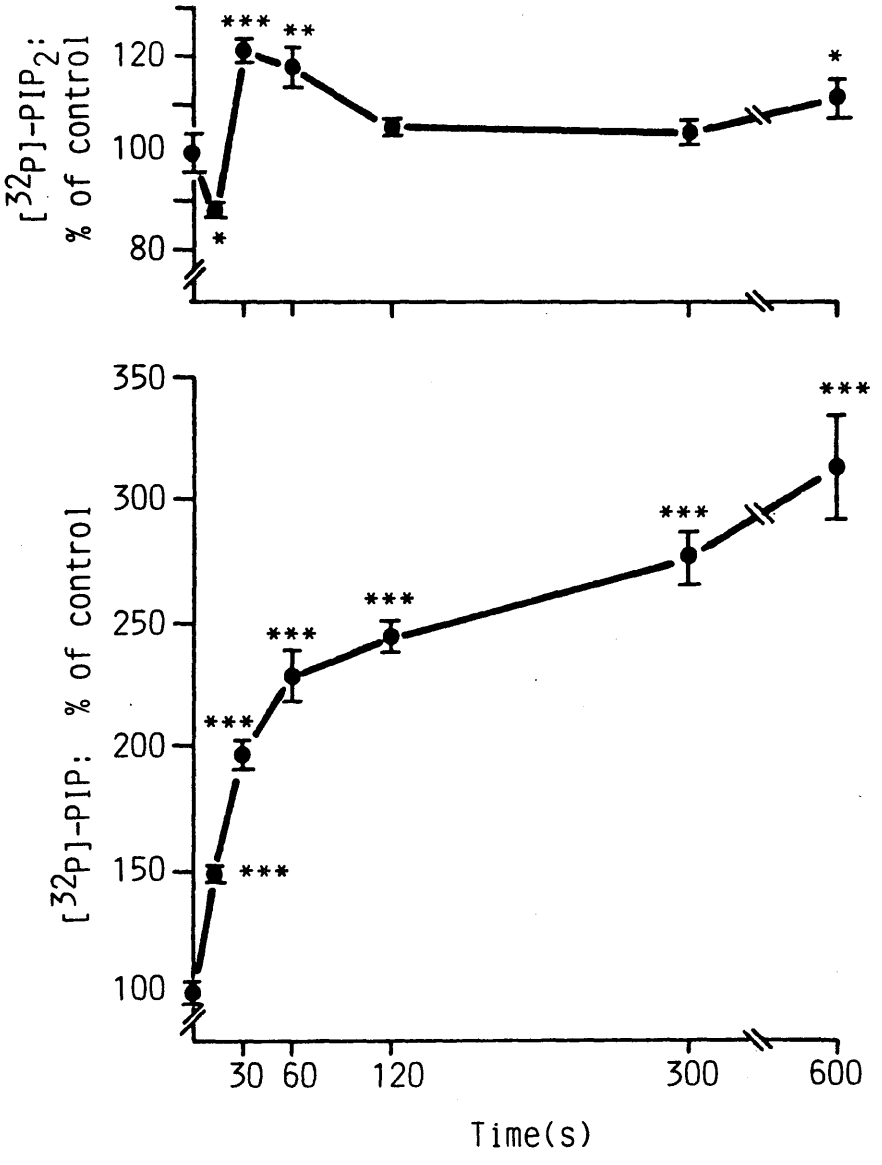

Figure 23. Time course of EP 171-induced changes in levels of $[^{32}P]$ poly phosphoinositides.

0.4 ml samples of $[^{32}P]$ P_i pulse chase labelled platelets were exposed to 625 nM EP 171 and the levels of $[^{32}P]$ PIP_2 (upper trace) and $[^{32}P]$ PIP (lower trace) were measured at the times indicated. The results are means $\pm$ SEM of triplicate determinations. The experiment shown is typical of 2 similar experiments. Analysis of data: *$P < 0.05$; **$P < 0.01$; ***$P < 0.001$.

$[^{32}P]$ Ptd OH formation induced by the three analogues. The rank order of potency is EP 171 > U44069 > Et PGH_2, with EC_{50}'s of 9 nM, 54 nM and approximately 1 μM respectively. As outlined in Figure 25, EP 171 (100 nM; 120s), U44069 (3 μM; 120s) and Et PGH_2 (30 μM; 120s) elicited $[^{32}P]$ Ptd OH formation of 840 ± 30%, 350 ± 23% and 179 ± 36% of the saline control respectively. However both U44069 and Et PGH_2 attenuated the EP 171 induced $[^{32}P]$ Ptd OH formation to 404 ± 21% and 353 ± 76% respectively. When agonist induced ATP secretion, expressed as a percentage of the maximum releasable ATP, is monitored (Figure 26), the order of potency is EP 171 > U44069 > Et PGH_2 (= 0). In addition, U44069 and Et PGH_2 inhibit EP 171 induced release of ATP from human platelets (Figure 27). These observations would perhaps be best explained were U44069, like Et PGH_2, to act as a partial agonist and EP 171 to act as a full agonist at the platelet Tx receptor.

1.4 Effect of thrombin on platelet phosphoinositide metabolism

Thrombin is probably the most widely studied platelet stimulatory agonist. It has been shown to elevate $[^{3}H]$ DAG (Rittenhouse-Simmons, 1979), decrease $[^{32}P]$ PIP_2 (Agranoff et al., 1983; Rendu et al., 1983), stimulate the formation of inositol phosphates (Siess & Binder, 1985), increase the amount of Ptd OH and decrease the amount of Ptd Ins (Broekman et al., 1980). The concentration response relationship for thrombin on Ptd OH in $[^{32}P]$ pre-labelled platelets is shown in Figure 28. Thrombin (0.01 - 3 U/ml (0.5 - 150 nM); 30s) elicited a concentration dependent formation of $[^{32}P]$ Ptd OH. The maximum concentration

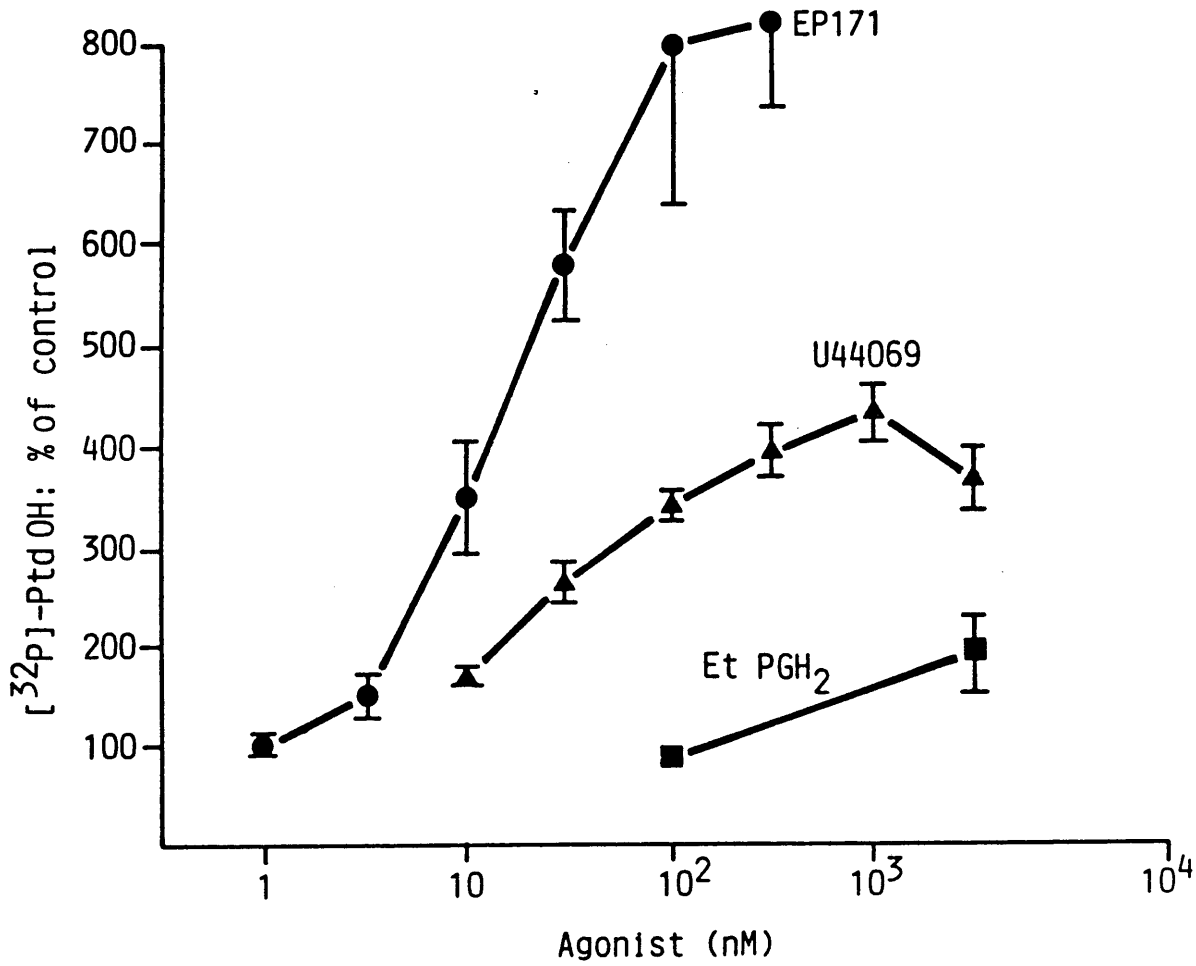

Figure 24. Concentration response curves for EP 171, U44069 and Et PGH_2 induced stimulation of $[^{32}P]$ Ptd OH formation.

0.4 ml samples of $[^{32}P]$ P_i prelabelled platelets were exposed to EP 171, U44069 or Et PGH_2 at the concentrations indicated. $[^{32}P]$ Ptd OH levels were measured 120s after agonist addition. (U44069 curve from MacIntyre et al., 1985).

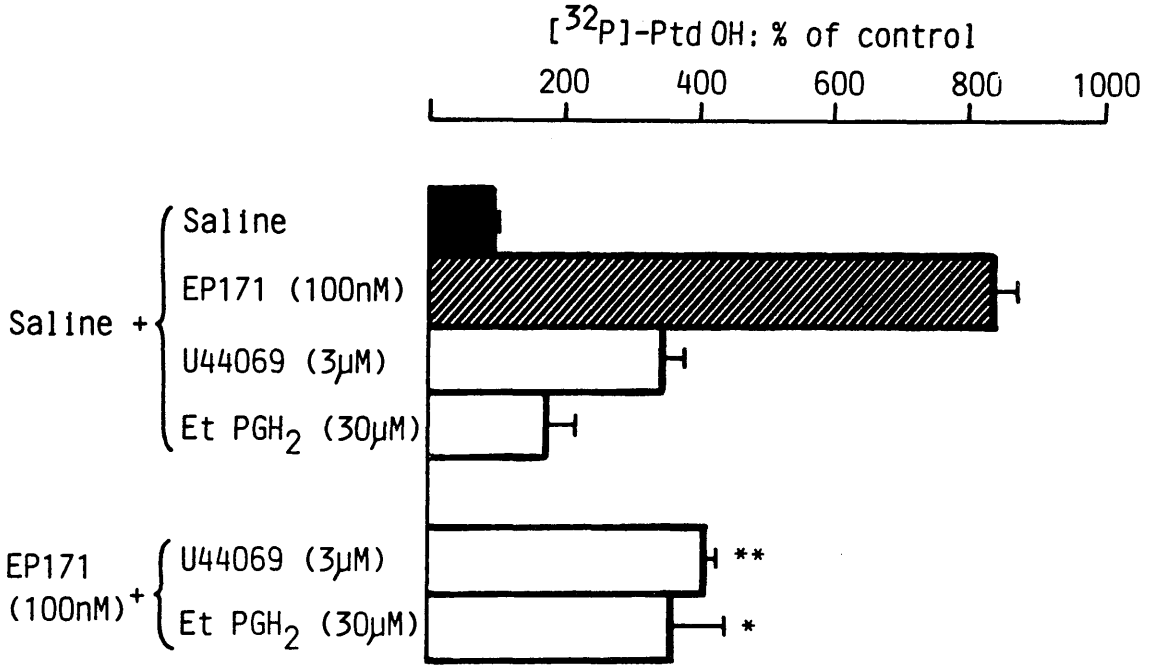

Figure 25. Effects of U44069 and Et PGH_2 on EP 171-induced $[^{32}P]$ Ptd OH formation.

0.4 ml samples of $[^{32}P]$ P_i prelabelled platelets were incubated with combinations of either saline or EP 171 (100 nM) and either saline, EP 171, U44069 or Et PGH_2 at the concentrations indicated. The levels of $[^{32}P]$ Ptd OH were measured 120s after the addition of the agonists. The results are means ± SEM of triplicate determinations. The experiment is typical of 3 similar experiments. Analysis of data (comparison with saline/EP 171): *$p < 0.01$; **$p < 0.001$.

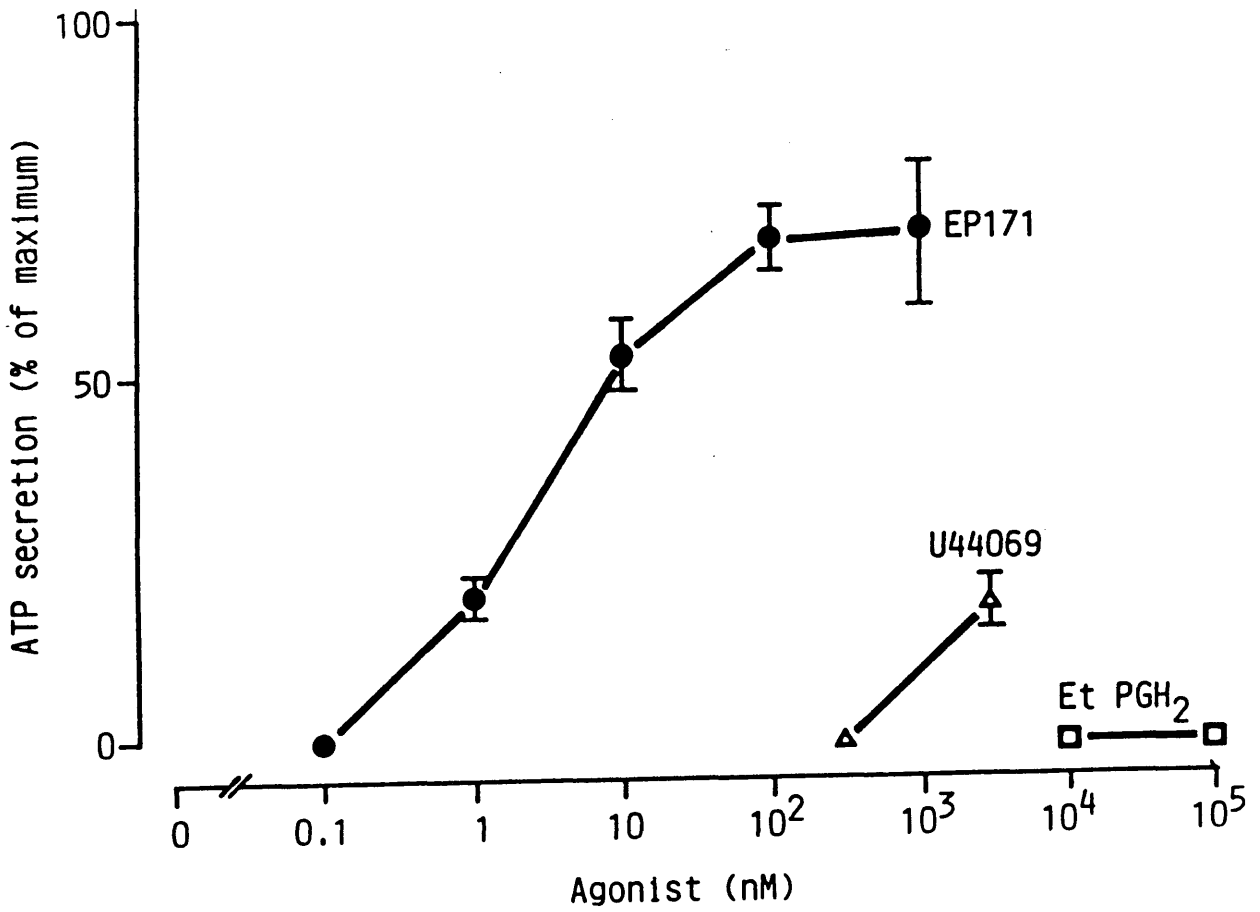

Figure 26. Concentration response curves for EP 171, U44069 and Et PGH_2 induced ATP release.

0.5 ml platelet samples were exposed to EP 171, U44069 or Et PGH_2 at the concentrations indicated. ATP release was measured by Lucifern-luciferase luminescence and expressed as a percentage of the maximum releasable ATP.

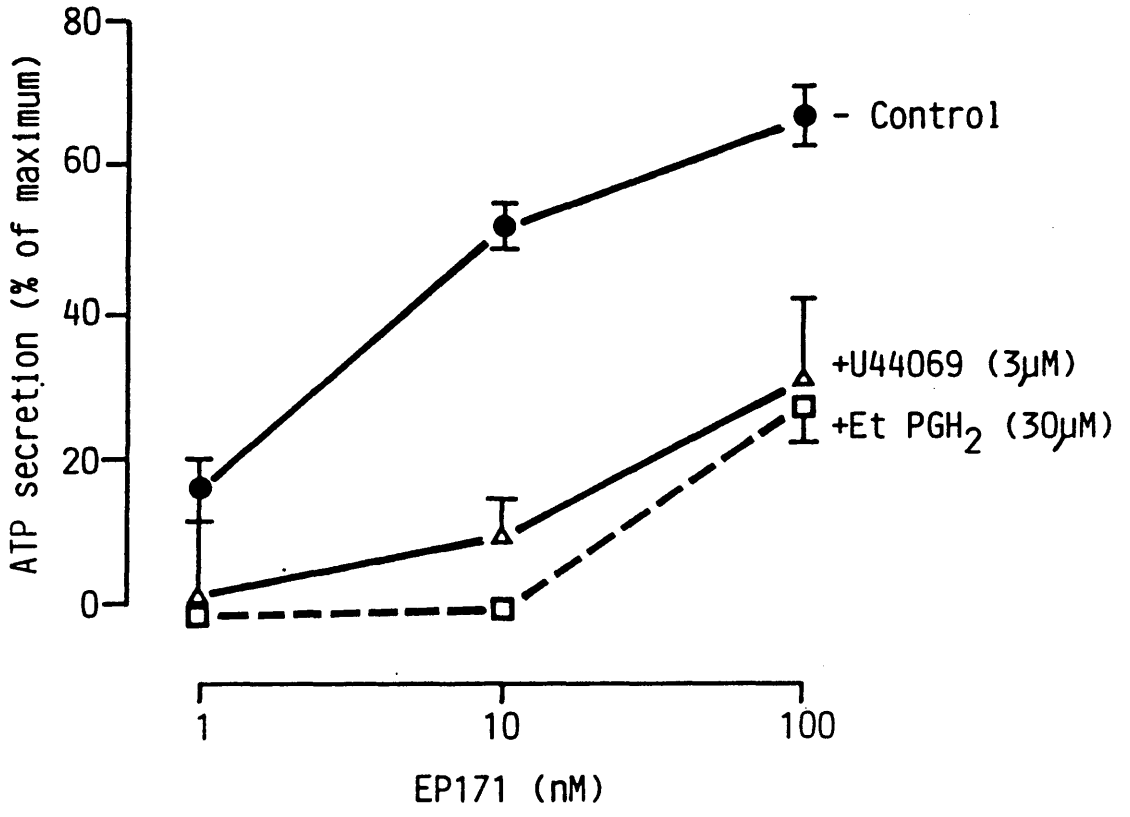

Figure 27. Effects of U44069 and Et PGH_2 on EP 171-induced ATP release.

0.5 ml platelet samples were challenged with EP 171 at the concentrations indicated and either saline (circles), 3 μM U44069 (triangles) or 30 μM Et PGH_2 (squares). ATP release was measured by Lucifern-luciferase luminescence and expressed as a percentage of the maximum releasable ATP.

of thrombin used was 3 U/ml and as the curve was still rising at this point it is impossible to assign a maximum level of stimulation and the EC_{50} can only be estimated to be > 0.1 U/ml (> 5 nM).

The maximum $[^{32}P]$ Ptd OH levels in response to thrombin (1 U/ml (50 nM))was achieved at 120s (2555 ± 121% of saline control) and this did not decline at 600s (Figure 29). This finding is relatively consistent with that of Agranoff, who demonstrated that the levels of $[^{32}P]$ Ptd OH were still rising at 120s, which was the latest time point he measured (Agranoff et al., 1983). The time course of the effects of thrombin (1 U/ml (50nM)) on $[^{32}P]$ -PIP_2 and -PIP levels in human platelets (Figure 30) shows a decrease in PIP_2 levels (to 74 ± 1% of the saline control) followed by an increase (to 128 ± 5% at 600s). There was a time dependent increase in the levels of PIP which reached 339 ± 11% at 600s following agonist addition.

1.5 Effect of agonists alone and in combination on $[^{32}P]$ Ptd OH formation

Results in Figure 28 and Figure 29 demonstrate that thrombin is by far the most efficacious and potent platelet stimulatory agonist at inducing $[^{32}P]$ Ptd OH formation. In order to investigate this further, thrombin induced $[^{32}P]$ Ptd OH was compared to that of a variety of compounds which may function as endogenous platelet agonists or mimics of endogenous platelet agonists in vivo. The platelets were challenged with high concentrations of the agonists both alone and when combined (Figure 31). ADP (1 μM; 30s) produced no $[^{32}P]$ Ptd OH formation,

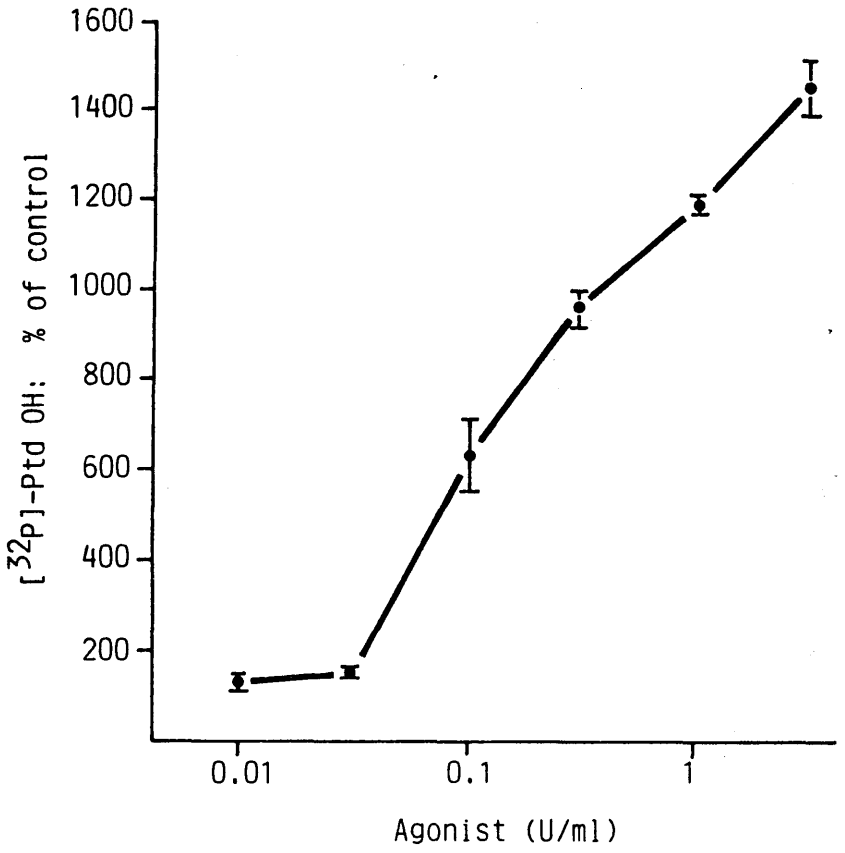

Figure 28. **Concentration response curve for thrombin-induced stimulation of $[^{32}P]$ Ptd OH formation.**

0.4 ml samples of $[^{32}P]$ P_i prelabelled platelets were exposed to thrombin at the concentrations indicated. $[^{32}P]$ Ptd OH levels measured 30s after agonist addition. The results are means ± SEM of triplicate determinations. The experiment is typical of 2 similar experiments.

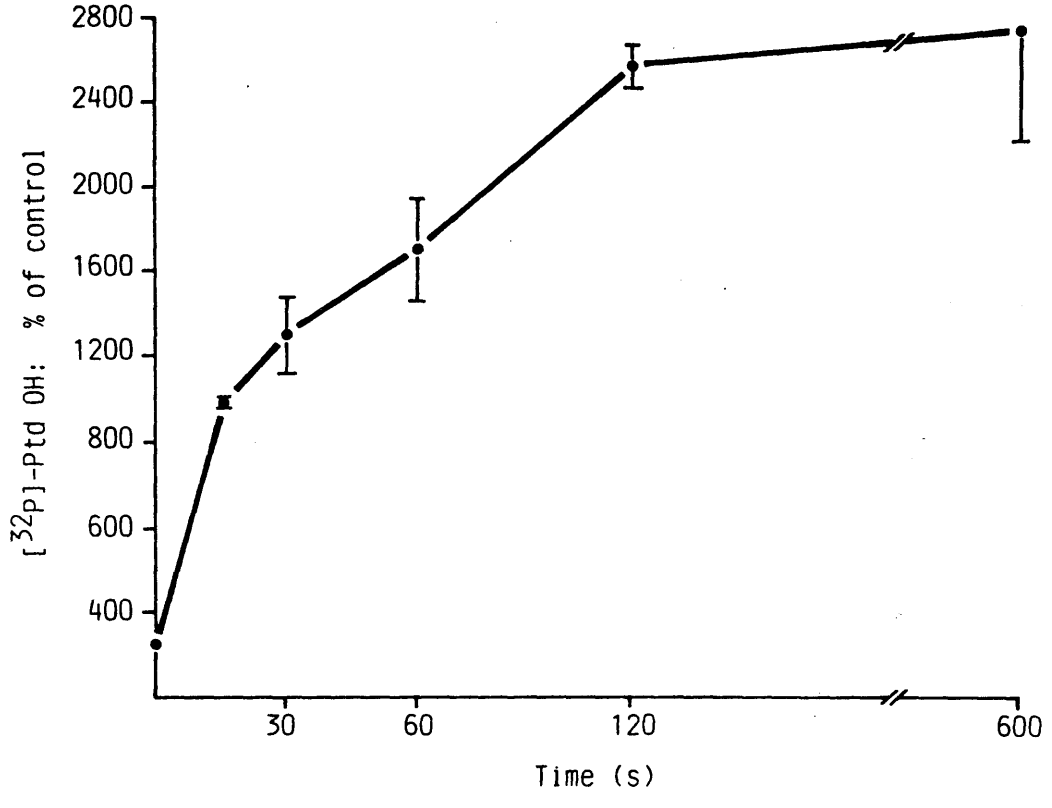

Figure 29. Time course of thrombin-induced stimulation of $[^{32}P]$ Ptd OH formation.

0.4 ml samples of $[^{32}P]$ P_i prelabelled platelets were exposed to 1U/ml (50 nM) thrombin and the levels of $[^{32}P]$ Ptd OH measured at the times indicated. The results are means ± SEM of triplicate determinations. The experiment is typical of 2 similar experiments.

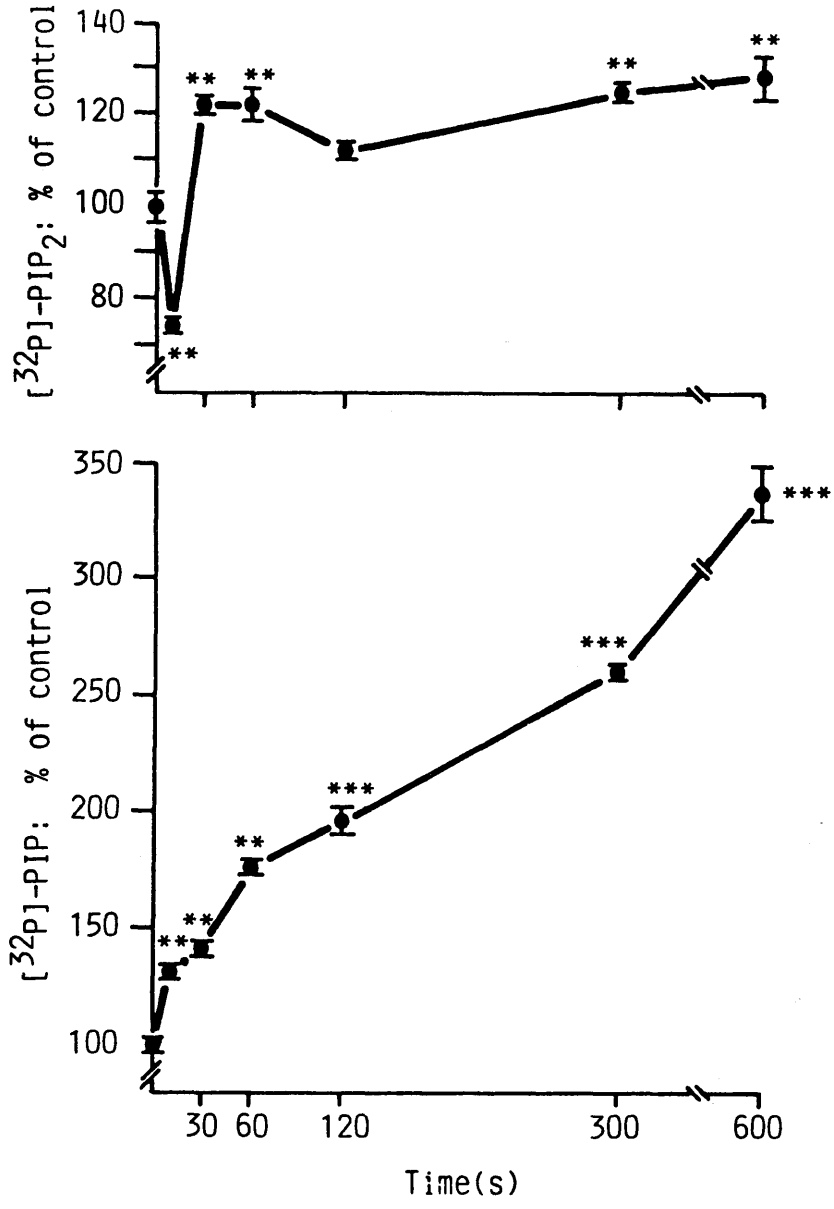

Figure 30. **Time course of thrombin-induced changes in levels of $[^{32}P]$ poly phosphoinosotides.**

0.4 ml samples of $[^{32}P]P_i$ pulse chased labelled platelets were exposed to 1U/ml (50 nM) thrombin and the levels of $[^{32}P]PIP_2$ (upper trace) and $[^{32}P]PIP$ (lower trace) measured at the times indicated. The results are means ± SEM of triplicate determinations. The experiment is typical of 3 similar experiments. Analysis of data: *$P < 0.05$; **$P < 0.01$; ***$P < 0.001$.

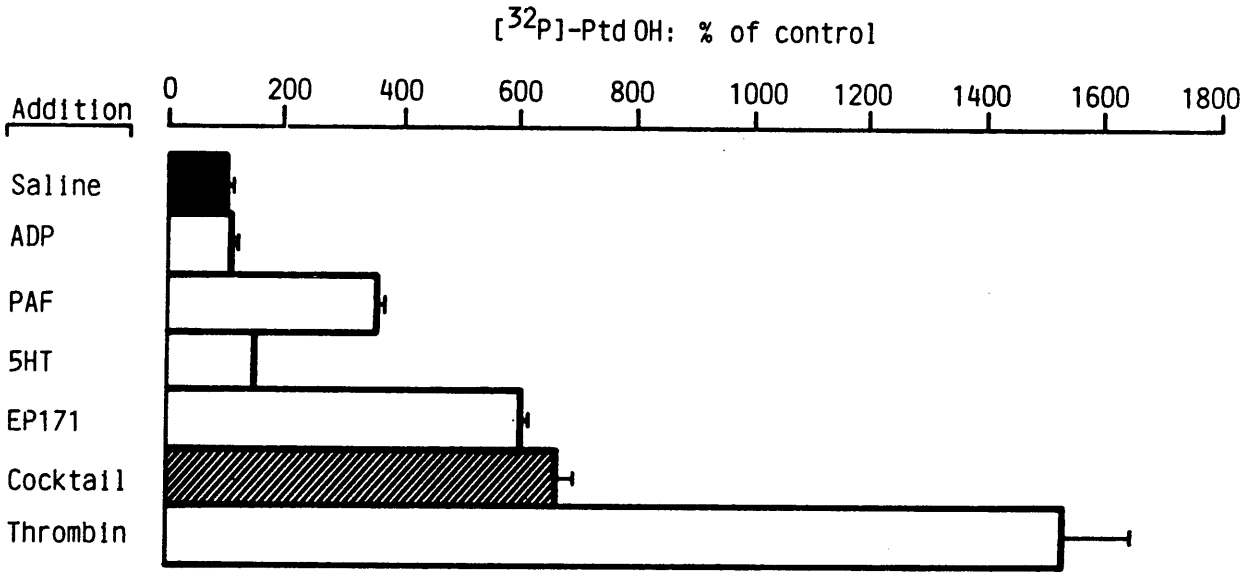

Figure 31. Effects of agonists alone and in combination on $[^{32}P]$ Ptd OH formation

0.4 ml samples of $[^{32}P]$ P_i prelabelled platelets were exposed to either saline, ADP (1 μM), PAF (180 nM), 5HT (10 μM) all for 30s, EP 171 (100 nM) for 120s, a cocktail containing the same concentrations of above agonists for the times indicated or thrombin (1U/ml; 50 nM) for 120s and the levels of $[^{32}P]$Ptd OH were measured. The results are means ± SEM of triplicate determinations. The experiment is typical of 4 similar experiments.

however PAF (180 nM; 30s), 5HT (10 μM; 30s) and EP 171 (100 nM; 120s) stimulated 356 ± 9%, 149 ± 2% and 607 ± 6% of the saline control respectively. The cocktail containing the same supra maximal agonist concentrations elicited a response of 663 ± 27% which is marginally more than the individual agonists alone elicit but considerably less than the 1529 ± 114% stimulation of $[^{32}P]$ Ptd OH evoked by thrombin (1 U/ml; 120s). Two possible explanations for this effect are that thrombin stimulates platelets via a proteolytic action and that some form of heterologous desensitisation occurs to prevent the cocktail of endogenous agonists from having an additive effect.

1.6 Effect of trypsin on platelet phosphoinositide metabolism

Trypsin, like thrombin, is a serine protease which has been shown to initiate the platelet functional response (Davey & Luscher, 1967). Comparison of thrombin and trypsin has shown that their enzyme saturating concentrations are 83 nM and 850 nM respectively (Martin et al., 1975).

Trypsin was used to examine the extent of the contribution that proteolytic activity made to the action of thrombin. Figure 32 shows the concentration response relationship for trypsin (10 nM - 10 μM; 120s) induced formation of $[^{32}P]$ Ptd OH. The maximal stimulation of $[^{32}P]$ Ptd OH formation is 3002 ± 70% and the estimated EC_{50} is 107 ± 39 nM. Thus proteases have the capacity to stimulate the formation of $[^{32}P]$ Ptd OH formation to a much greater extent than the classical receptor directed agonists. Whether such an effect is due solely to proteolytic activity or is mediated in part by some form of receptor-

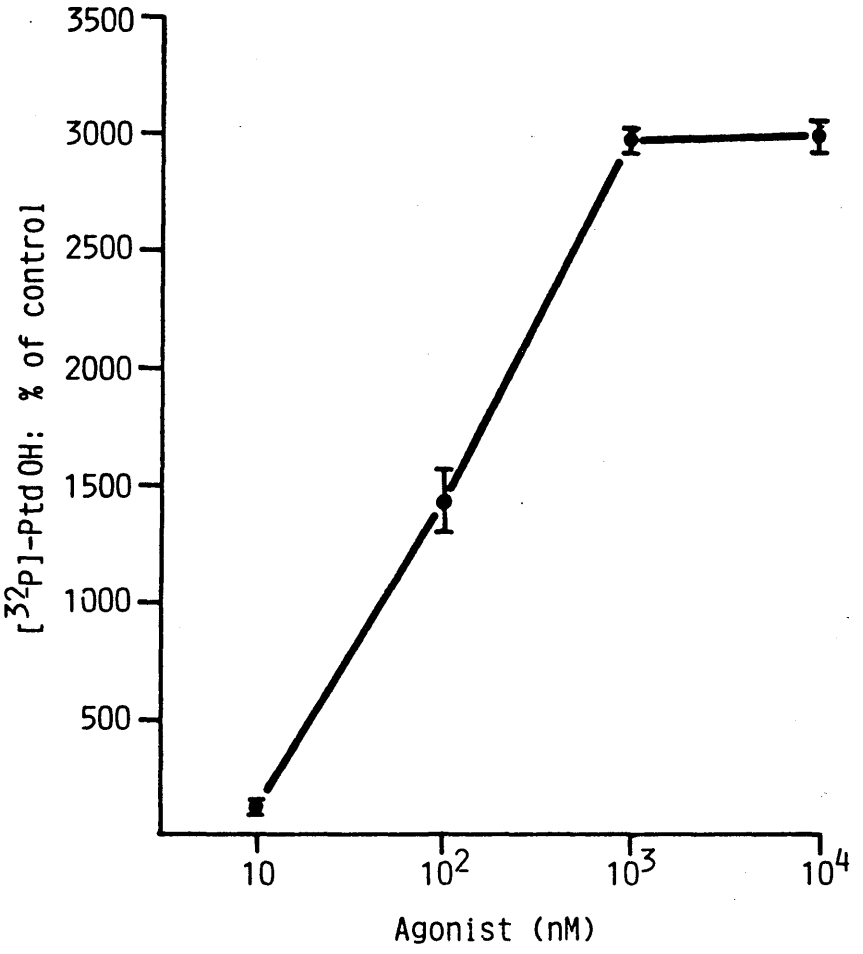

Figure 32. Concentration response curve for trypsin-induced stimulation of $[^{32}P]$ Ptd OH formation.

0.4 ml samples of $[^{32}P]$ P_i prelabelled platelets were exposed to trypsin at the concentration indicated. $[^{32}P]$ Ptd OH levels were measured 120s after agonist addition. The results are means $\pm$ SEM of triplicate determinations. The experiment is typical of 3 similar experiments.

activated process cannot be estimated on the available evidence.

1.7 Effect of trypsin and EP 171 on platelet phosphoinositide metabolism

To examine possible additive effects of protease and receptor mediated phosphoinositide hydrolysis, trypsin and EP 171 were co-added to platelets. The extent of $[^{32}P]$ Ptd OH formation evoked by trypsin (1 μM; 120s) alone is 3058 ± 76% and by EP 171 (100 nM; 120s) alone 722 ± 2%. In both cases data is expressed relative to the saline control. When these agonists were combined the resultant extent of $[^{32}P]$ Ptd OH formation increased to 3600 ± 55% of the saline control. Thus the combination is significantly more effective than either trypsin or EP 171 alone: indeed the potentiation appears to be almost exactly additive (Figure 33). This suggests that mechanistically it is possible for a dual stimulation of human platelets via both a receptor-activated (typified by EP 171) and a protease (typified by trypsin). Pretreatment with the protease inhibitor leupeptin (100 μg/ml; 60s), inhibits the trypsin-induced formation of $[^{32}P]$ Ptd OH both alone and in combination with EP 171 (Figure 34). It is therefore possible that proteases stimulate platelets by acting by a different process from other types of agonists, although its precise mechanism of action cannot be elucidated from this data. The proteases (thrombin and trypsin) clearly differ qualitatively and quantitatively from other agonists in their coupling mechanism to phospholipase C catalysed phosphoinositide hydrolysis.

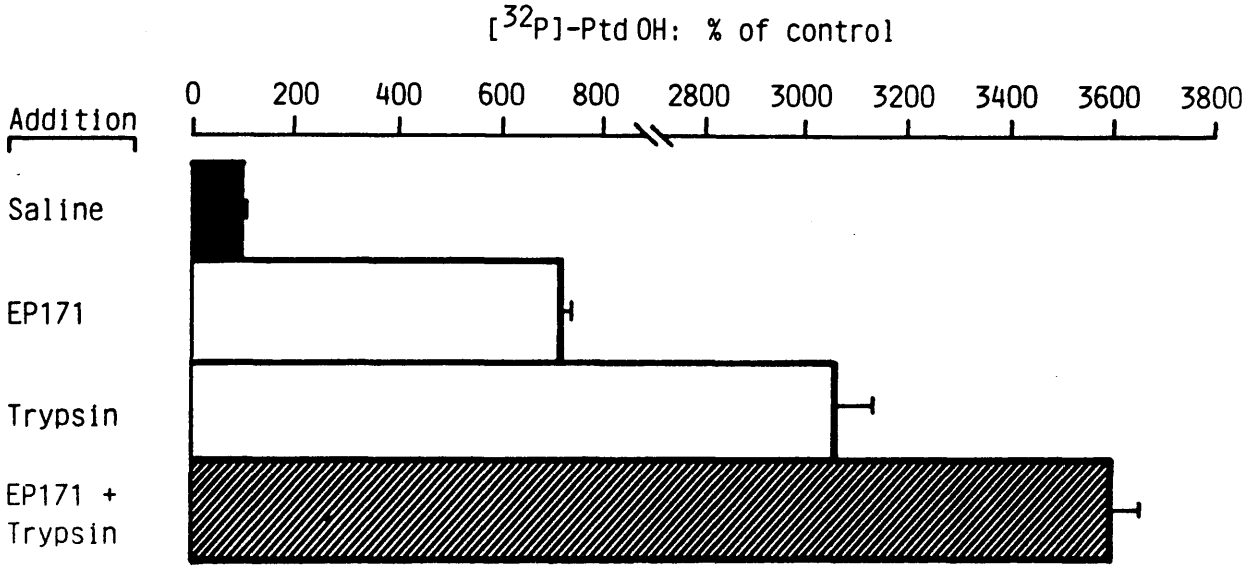

Figure 33. EP 171 and trypsin stimulation of $[^{32}P]$ Ptd OH formation.

0.4 ml samples of $[^{32}P]$ P_i prelabelled platelets were exposed to either saline, EP 171 (100 nM), trypsin (1 μM), or a combination of EP 171 (100 nM) and trypsin (1 μM). $[^{32}P]$ Ptd OH levels were measured 120s after addition of the agonist(s). The results are means ± SEM of triplicate determinations. The experiment is typical of 3 similar experiments. Analysis of data: EP 171 + trypsin versus:- 1) trypsin $p < 0.01$; 2) EP 171 $p < 0.001$.

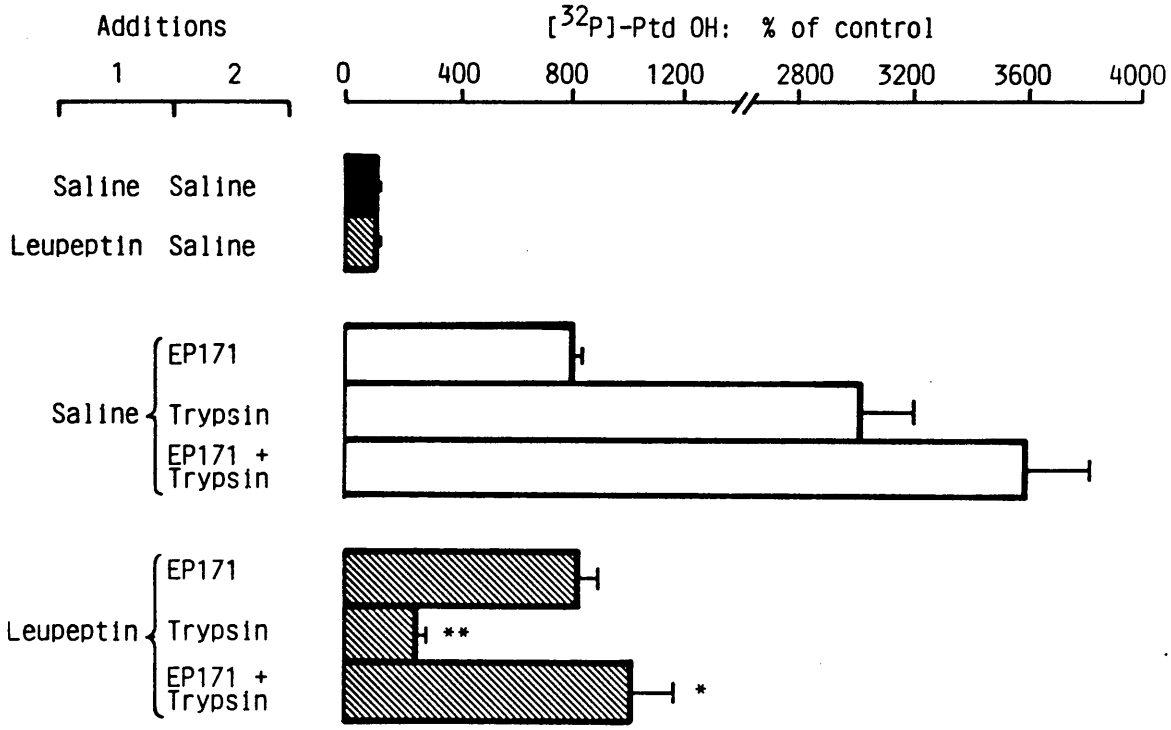

Figure 34. Effects of leupeptin on EP 171 and trypsin-induced stimulation of $[^{32}P]$ Ptd OH formation.

0.4 ml samples of $[^{32}P]$ P_i prelabelled platelets were pre-incubated for 60s with either saline (open bars) or 100 µg/ml leupeptin (hatched bars), prior to the addition of either saline, EP 171 (100 nM), trypsin (1 µM) or a combination of EP 171 (100 nM) and trypsin (1 µM). $[^{32}P]$ Ptd OH levels were measured 120s after addition of agonist(s). The results are means ± SEM of triplicate determinations. The experiment is typical of 2 similar experiments. Analysis of data: *$p < 0.01$; **$p < 0.001$.

1.8 Effect of PMA on platelet phosphoinositide metabolism

The phorbol ester class of compound have been shown to stimulate human platelets by a direct activation of protein kinase C and therefore should bypass receptor mediated phosphoinositide hydrolysis. Figure 35 demonstrates the effects of the phorbol ester PMA on $[^{32}P]$-Ptd OH and -Ptd Ins levels. PMA (100 nM) evoked no alteration in the labelling of Ptd OH up to 600s following its addition. This suggests that, as expected, PMA does not stimulate phospholipase C activity. In contrast however 120s to 600s after challenge, PMA caused a decrease in the levels of $[^{32}P]$ Ptd Ins when compared to the DMSO control indicative of some effect by PMA on phosphoinositide metabolism.

To further investigate this, the effect of PMA on $[^{32}P]$ -PIP_2 and -PIP were examined. PMA (100 nM) did not elicit an initial decrease in the levels of PIP_2, as for example EP 171 (Figure 23) and thrombin (Figure 30) do, but did stimulate increased levels of both $[^{32}P]$ -PIP_2 and -PIP (Figure 36). This increase in the levels of the poly phosphoinositides occurs late in response to PMA (60s for PIP; 120s for PIP_2) unlike the corresponding increase in response to receptor directed agonists such as EP 171 (Figure 23) and thrombin (Figure 30).

1.9 Summary

These results demonstrate that thrombin, EP 171 and trypsin but not ADP and PMA induced stimulation of the human platelet is associated with and may be mediated by inositol phospholipid hydrolysis.

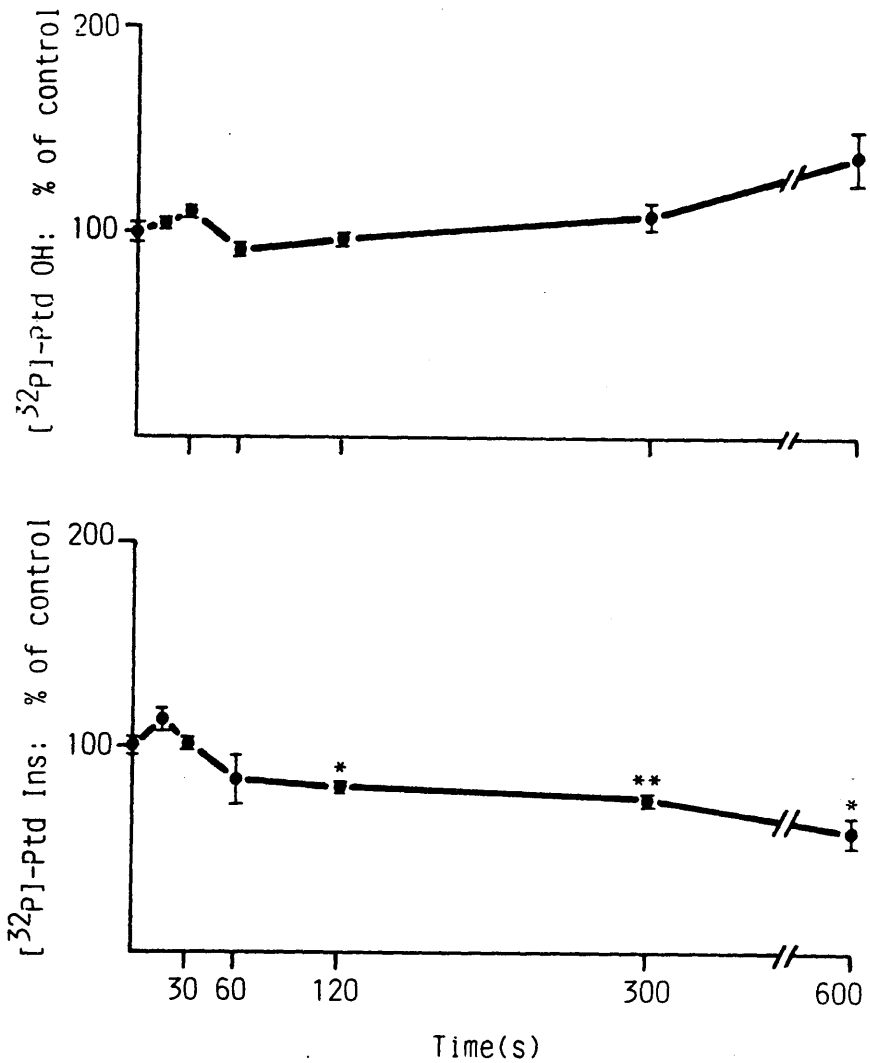

Figure 35. Time course of PMA-induced stimulation of $[^{32}P]$ Ptd OH formation.

0.4 ml samples of $[^{32}P]$ P_i prelabelled platelets were exposed to 100 nM PMA and the levels of $[^{32}P]$Ptd OH measured at the times indicated. The results are means $\pm$ SEM of triplicate determinations. The experiment is typical of 3 similar experiments. Analysis of data: *$p < 0.05$; **$p < 0.01$.

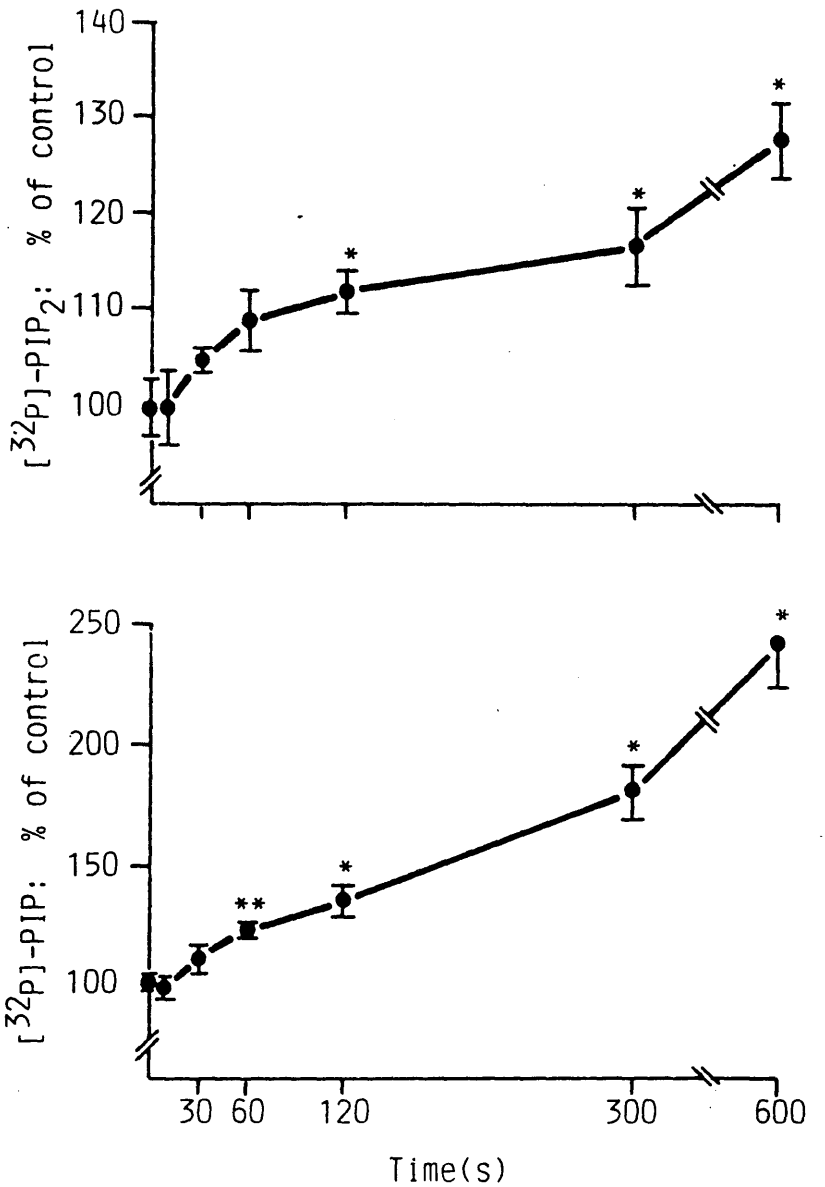

Figure 36. Time course of PMA-induced changes in levels of $[^{32}P]$ poly phosphoinositides

0.4 ml samples of $[^{32}P]_i$ prelabelled platelets were exposed to 100 nM PMA and the levels of $[^{32}P]$ PIP_2 (upper trace) and $[^{32}P]$ PIP (lower trace) were measured at the times indicated. The results are means ± SEM of triplicate determinations. The experiment is typical of 3 similar experiments. Analysis of data: *$p < 0.05$; **$p < 0.01$.

EP 171 is a more potent agonist at the platelet Tx receptor than, the previously reported, U44069 (Pollock et al., 1984). Indeed it appears that EP 171 is a full agonist and U44069 a partial agonist at this receptor.

Thrombin is the most potent and efficacious agonist so far examined. There are at least two possible explanations for the inability of combinations of other agonists to match the level of thrombin induced $\left[^{32}P\right]$ Ptd OH formation. Firstly thrombin may stimulate platelets by both receptor mediated and proteolytic mechanisms, and the effects of trypsin indicate that proteases can stimulate $\left[^{32}P\right]$ Ptd OH formation. Secondly there may be some form of desensitisation amongst the other agonists.

2. Mechanisms underlying desensitisation

In this section I examine the inter-relationships between phosphoinositide metabolism and elevation of $\left[Ca^{2+}\right]_i$. In particular the regulation of $\left[Ca^{2+}\right]_i$ by agonist concentration, by adenylate and guanylate cyclase stimulants and by phorbol esters is compared to the effects of the same processes on platelet phosphoinositide metabolism. In addition the possibility that certain endogenous agents can act as mediators of desensitisation was examined.

2.1 Effect of agonist concentration on platelet $\left[Ca^{2+}\right]_i$

When an agonist is added to platelets pre-incubated with Quin 2 it elicits a concentration dependent increase in fluorescence which is readily calibrated to report $\left[Ca^{2+}\right]_i$

and increases in $[Ca^{2+}]_i$. The resting $[Ca^{2+}]_i$ in human platelets is 90 ± 3 nM (mean ± SEM). A typical experimental fluorescence record for PAF (1.8 - 180 nM) is shown in Figure 37. As the agonist concentration increased, the rate and extent of the resultant fluorescence (and so $[Ca^{2+}]_i$) also increased until a maximum value (400 - 700 nM in different experiments) was attained. Thereafter the fluorescence declined towards the resting value. The rate of decline of the signal also varied with the agonist concentration; the response elicited by the high concentration decayed more rapidly than that elicited by the lower concentrations.

2.2 Effect of agonist concentration on platelet phosphoinositide metabolism

Inositol phospholipid hydrolysis mechanistically is believed to underlie elevated $[Ca^{2+}]_i$. Consequently the effects of agonist concentration on the profile of the time course of agonist-induced $[^{32}P]$ Ptd OH formation was examined. The time courses of supra maximal (1.8 μM) and threshold (18 nM) PAF at eliciting the formation of $[^{32}P]$ Ptd OH are compared in Figure 38. The supra maximal PAF concentration displays a rapid elevation of Ptd OH which plateaus at 30 - 60s and this was followed by a rapid decrease. In contrast 18 nM PAF stimulated a much slower rise and fall in Ptd OH levels which was significantly elevated at 120 - 300s only. This mirrors the situation observed when the time course of agonist-induced elevation of $[Ca^{2+}]_i$ is monitored, and may suggest that some form of desensitisation occurs.

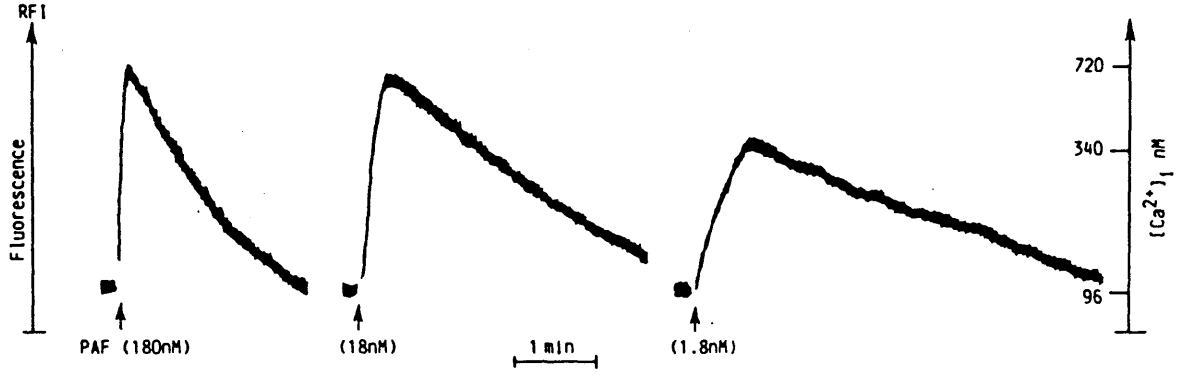

Figure 37. Effect of concentration on PAF-induced elevation of $[Ca^{2+}]_i$

2.0 ml samples of Quin 2 labelled platelets were exposed to PAF at the concentrations indicated. Fluorescence responses were monitored after agonist addition and the appropriate $[Ca^{2+}]_i$ calibration scale is shown on the right of the fluorescence record. The experiment shown is typical of 3 similar experiments.

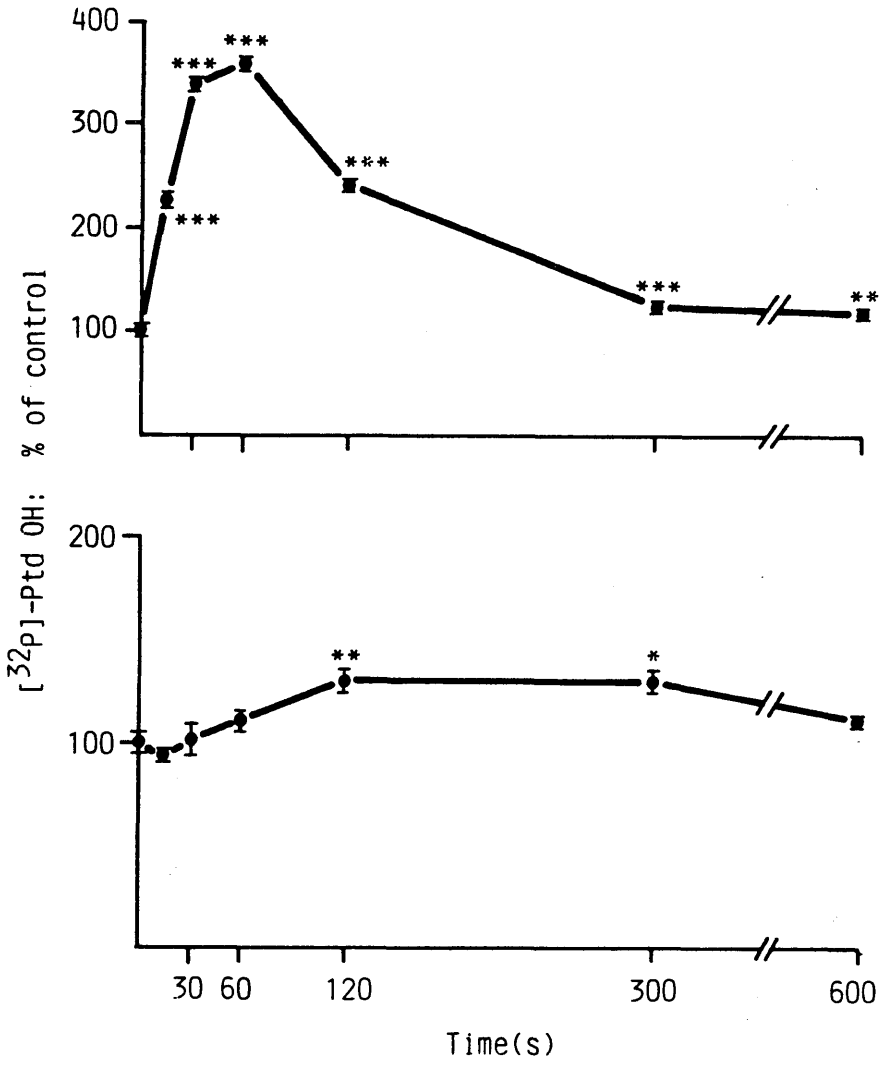

Figure 38. Effect of concentration on PAF-induced stimulation of $[^{32}P]$ Ptd OH formation.

0.4 ml samples of $[^{32}P]P_i$ prelabelled platelets were exposed to 1.8 μM PAF (upper trace) or to 18 nM PAF (lower trace) and the levels of $[^{32}P]$ Ptd OH measured at the times indicated. The results are means ± SEM of triplicate determinations. The experiment is typical of 2 similar experiments at each concentration. Analysis of data: *$p < 0.05$; **$p < 0.01$; ***$p < 0.001$.

2.3 Effect of second addition of agonist on platelet phosphoinositide metabolism

To further investigate possible desensitisation in phosphoinositide response, a supra maximal PAF concentration (1.8 μM) was added to $[^{32}P]$ pre-labelled platelets and this elevated the $[^{32}P]$ Ptd OH levels to 476 ± 145% of the saline control at 30s (Figure 39). The platelets were subsequently challenged with PAF (1.8 μM; 30s) and this second addition failed to elicit further formation of Ptd OH. However were vasopressin added as the second agonist then further Ptd OH formation results when compared to the time matched saline control. The amount of vasopressin induced Ptd OH formation is less than that formed when this agonist is added alone, and is much less than that elicited by thrombin (Figure 39). This is perhaps indicative of heterologous desensitisation and clearly suggests that desensitisation is not due to limited levels of inositol phospholipids.

As desensitisation is not due to the substrate availability, I examined the possibility that endogenous compounds exist which act as mediators of desensitisation. Putative candidates for such a function are the cyclic nucleotides, cAMP and cGMP as elevated levels of both compounds have been shown to inhibit platelet function (Haslam et al., 1980; Glusa et al., 1974).

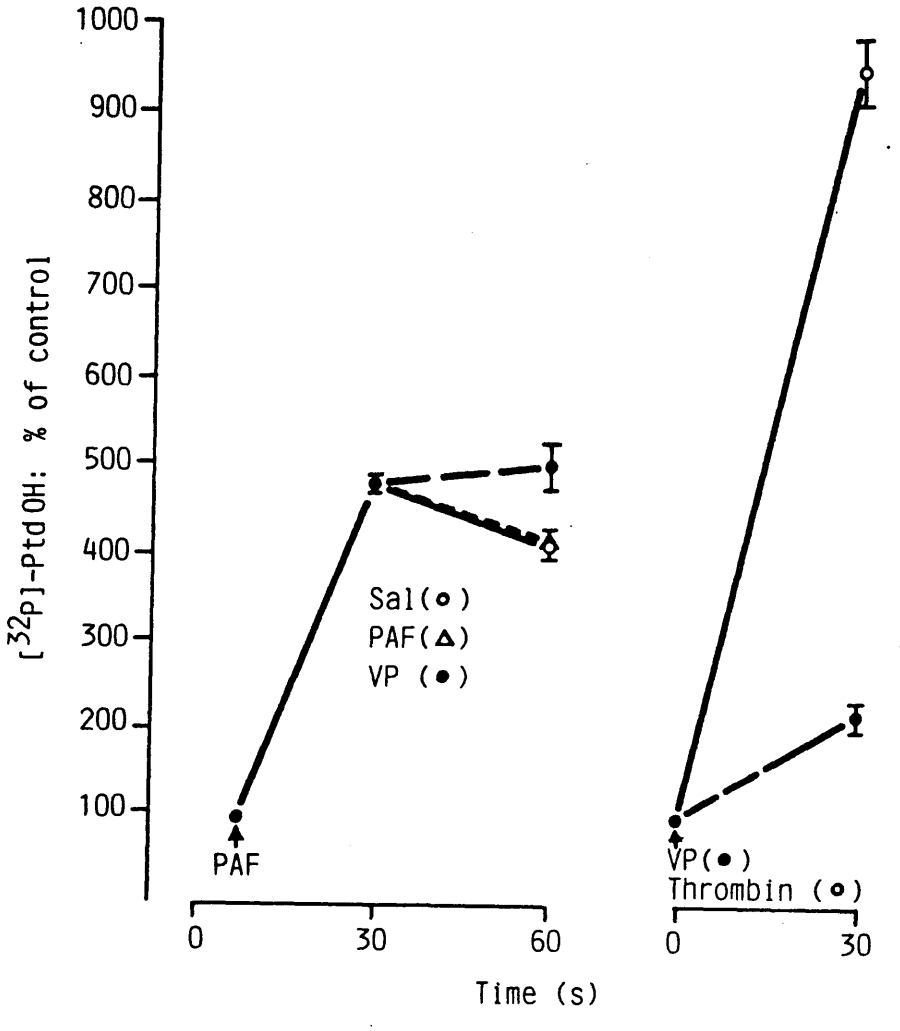

Figure 39. Desensitisation of agonist-induced $[^{32}P]$ Ptd OH formation.

0.4 ml samples of $[^{32}P]P_i$ prelabelled platelets were exposed to 1.8 μM PAF and the levels of $[^{32}P]$ Ptd OH measured at the times indicated. At 30s the platelets were again challenged with either saline (open circles), 1.8 μM PAF (open triangles) or 1 μM vasopressin (closed circles) and the levels of $[^{32}P]$ Ptd OH measured at 60s. Vasopressin (1 μM) and thrombin (1U/ml (50 nM)) controls, which are not desensitised, are depicted for comparison. The results are means ± SEM of triplicate determinations. The experiment is typical of 3 similar experiments.

2.4 Effects of agents that influence cyclic nucleotides on platelet $[Ca^{2+}]_i$

In order to examine possible mechanisms of desensitisation the effects of cyclic nucleotides, which are potent inhibitors of platelet function, on $[Ca^{2+}]_i$ were investigated. The adenylate cyclase stimulants PGI_2 and PGD_2 (both < 1 μM; 120s), the guanylate cyclase stimulant NaNP (< 10 μM; 120s) and the stable cyclic nucleotide analogues 8Br cAMP and 8Br cGMP (both < 1 mM; 300s) were added to Quin 2 labelled platelets prior to challenge with sub-maximal PAF (10 - 100 nM). Each agent inhibited, in a concentration dependent manner, the agonist induced elevation of $[Ca^{2+}]_i$ (Figure 40) although none of the compounds altered basal $[Ca^{2+}]_i$.

2.5 Effect of agents that influence cyclic nucleotides on platelet phosphoinositide metabolism

Similar studies were carried out on platelet phosphoinositide metabolism. PGD_2, NaNP (both < 1 μM; 90s) and the stable cyclic nucleotide analogues 8Br cAMP and 8Br cGMP (both 1 mM; 300s) had no effect on basal $[^{32}P]$ Ptd OH levels (Figure 41). In contrast PGD_2 (3 - 300 nM; 90s) inhibits PAF (18 nM; 30s; Figure 42) and vasopressin (100 nM; 30s; Figure 43) induced formation of $[^{32}P]$ Ptd OH with I_{50} values of around 10 nM and 30 nM respectively. Similarly NaNP (0.3 nM - 3 μM; 90s) inhibits PAF (18 nM; 30s) induced $[^{32}P]$ Ptd OH formation in a concentration dependent manner with an I_{50} of around 30 nM (Figure 44). Were cAMP and/or cGMP to be produced by platelets following challenge with stimulatory agonists, then both have the capacity to serve as endogenous mediators of desensitisation.

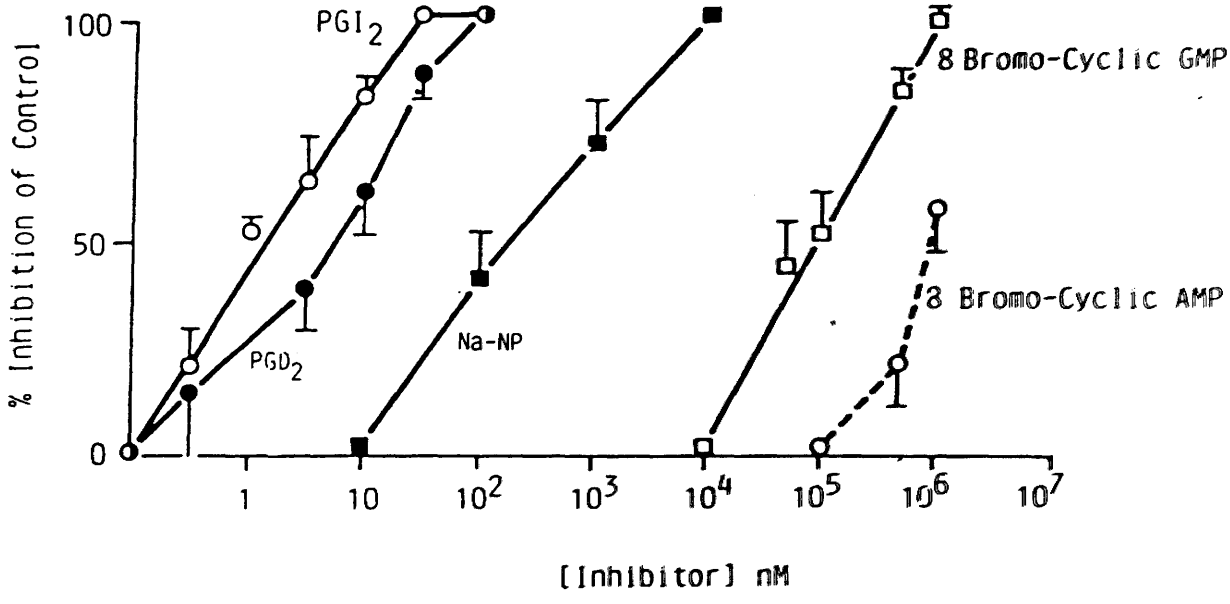

Figure 40. Effects of agents which influence cyclic nucleotides on PAF-induced elevation of $[Ca^{2+}]_i$

2.0 ml of Quin 2 labelled platelets were pre-incubated with PGI_2, PGD_2, NaNP, 8 Br cAMP, 8 Br cGMP or the appropriate vehicle at the concentration indicated prior to the addition of a sub-maximal concentration of PAF (10_2- 100 nM). Fluorescence responses were monitored, calibrated for $[Ca^{2+}]_i$ and are expressed as inhibition of the PAF (+ vehicle) control.

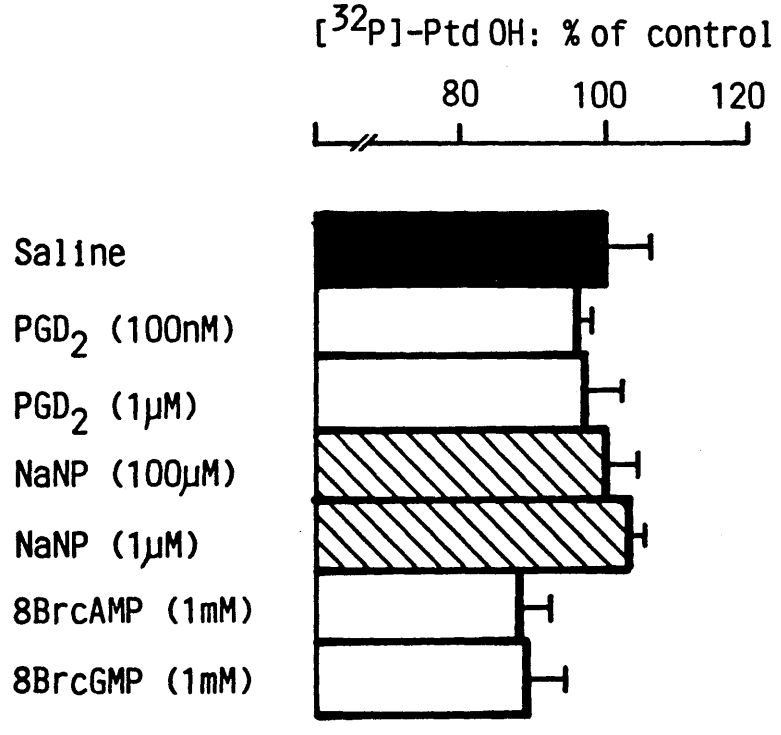

Figure 41. Effects of agents which influence cyclic nucleotides on $[^{32}P]$ Ptd OH levels.

0.4 ml samples of $[^{32}P]$ P_i prelabelled platelets were exposed either to PGD_2 or NaNP for 90s or to 8 Br cAMP or cGMP for 300s at the concentrations indicated. $[^{32}P]$ Ptd OH levels were measured at the appropriate times. The results are means ± SEM of triplicate determinations. The experiment is typical of 2-4 similar experiments.

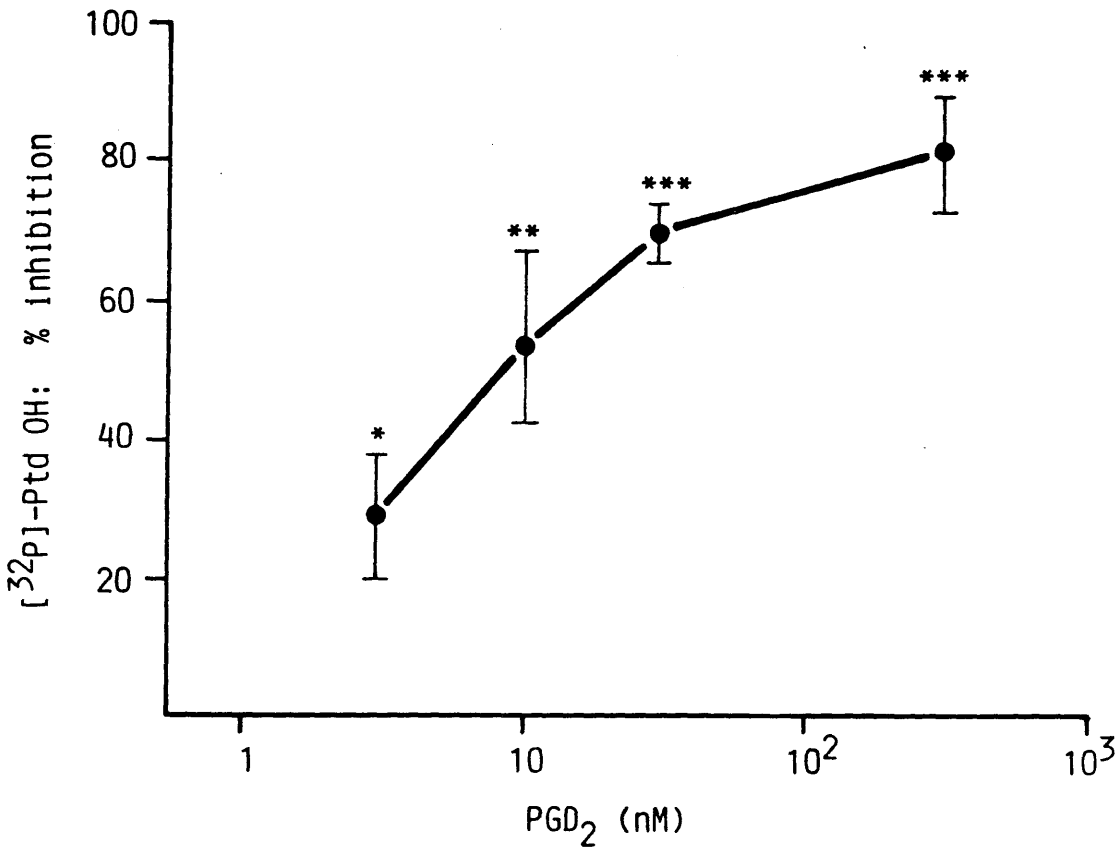

Figure 42. Effect of PGD_2 on PAF-induced stimulation of $[^{32}P]$ Ptd OH formation.

0.4 ml samples of $[^{32}P]$ P_i prelabelled platelets were pre-incubated for 90s with PGD_2, at the concentrations indicated, or with the saline control. Samples were then challenged with 18 nM PAF and $[^{32}P]$ Ptd OH measured 30s after agonist addition. The results are means ± SEM of 2 similar experiments each performed in triplicate. Analysis of data (expressed as inhibition of PAF (+ saline) response): *$p < 0.05$; **$p < 0.01$; ***$p < 0.001$.

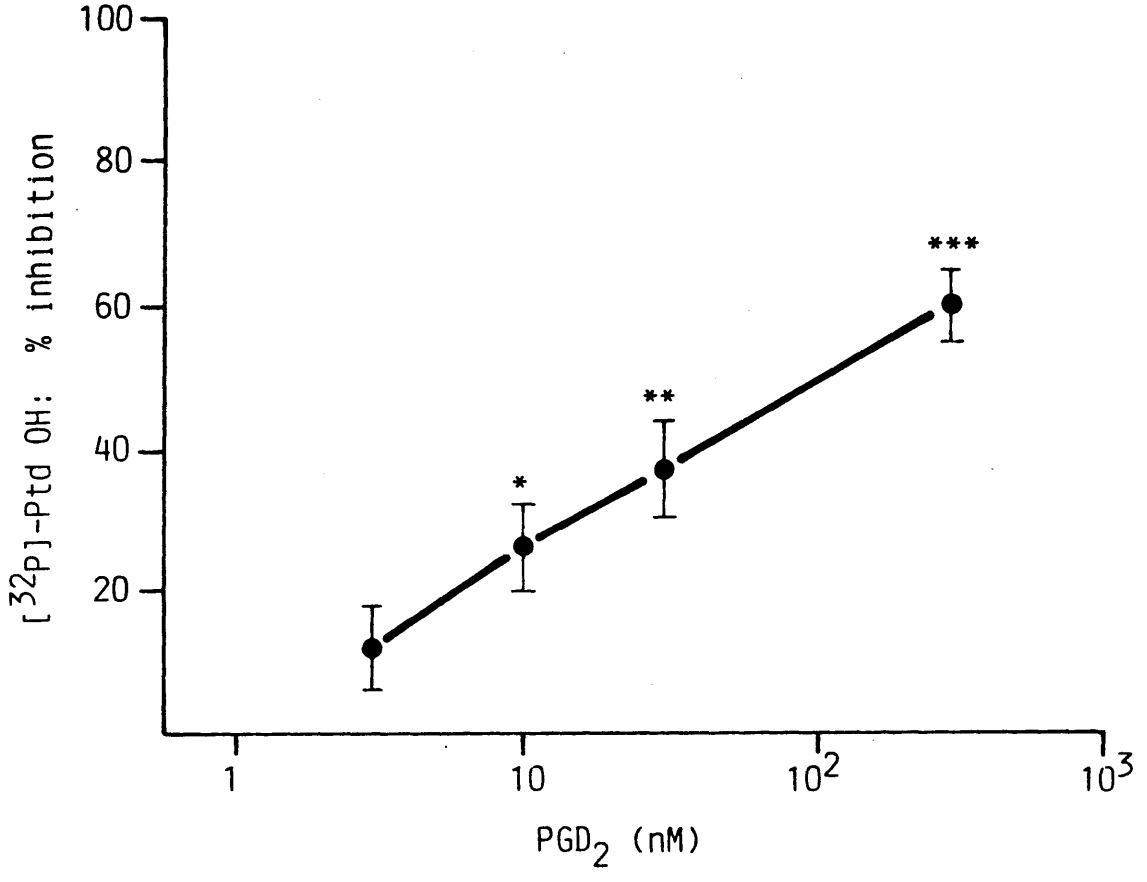

Figure 43. Effect of PGD_2 on vasopressin-induced stimulation of $[^{32}P]$ Ptd OH formation.

0.4 ml samples of $[^{32}P]$ P_i prelabelled platelets were pre-incubated for 90s with PGD_2, at the concentrations indicated, or with the saline control. Samples were then challenged with 100 nM vasopressin and $[^{32}P]$ Ptd OH measured 30s after agonist addition. The results are means ± SEM of 3 similar experiments each performed in triplicate. Analysis of data (expressed as inhibition of vasopressin (+ saline) response): *$p < 0.01$; **$p < 0.005$; ***$p < 0.001$.

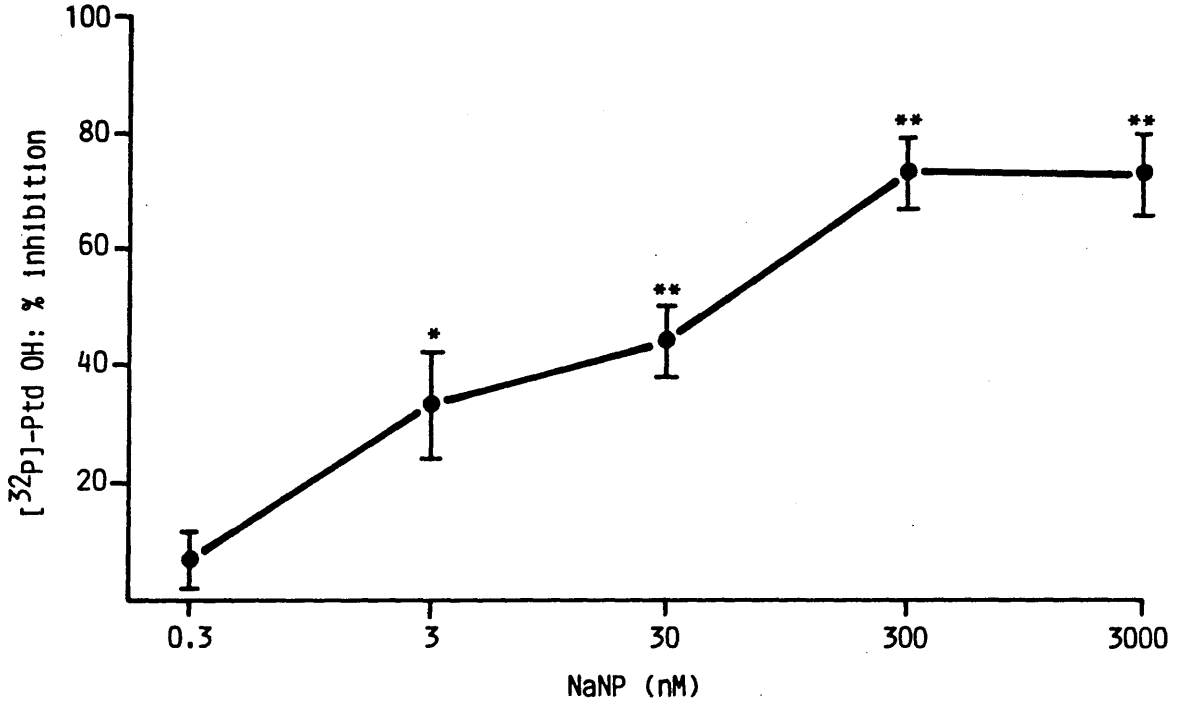

Figure 44. Effect of NaNP on PAF-induced stimulation of $[^{32}P]$ Ptd OH formation.

0.4 ml samples of $[^{32}P]$ P_i prelabelled platelets were pre-incubated for 90s with NaNP, at the concentrations indicated, or with the saline control. Samples were then challenged with 18 nM PAF and $[^{32}P]$ Ptd OH levels measured 30s after agonist addition. The results are means $\pm$ SEM of 3 similar experiments each performed in triplicate. Analysis of data (expressed as inhibition PAF (+ saline) response): *$p < 0.05$; **$p < 0.001$.

Studies in neutrophils have indicated that the protein kinase C stimulants, the phorbol esters, activate an ATP-dependent calcium extrusion process (Mottola & Romeo, 1982) and attenuate an agonist-induced elevation in $[Ca^{2+}]_i$. The phorbol esters PMA and PDBu were used to examine whether protein kinase C activation in human platelets have similar pronounced inhibitory effect on the stimulatory transduction process. The inactive phorbol analogue, 4βphorbol, which is structurally similar to phorbol esters but does not activate protein kinase C (Castagna et al., 1982), was used as the negative control in these studies.

2.6 Effect of phorbol esters on agonist induced elevation of $[Ca^{2+}]_i$

Using Quin 2 labelled platelets thrombin (0.025U/ml (1.25 nM) vasopressin (50 nM) and PAF (10 nM) caused an elevation of $[Ca^{2+}]_i$ from a resting value of 90 ± 3 nM to a maximum of 510 ± 180 nM, 420 ± 80 nM and 480 ± 100 nM respectively. When incubated with platelets for 120s, neither PMA nor 4βphorbol (both < 1.6 μM) altered resting $[Ca^{2+}]_i$. However PMA (16 - 160 nM) but not 4βphorbol inhibited in a concentration dependent manner the elevation of $[Ca^{2+}]_i$ induced by all three agonists (Figure 45).

2.7 Effect of phorbol esters on agonist induced platelet phosphoinositide metabolism

PMA (1.6 μM; 120s) had no effect on platelet $[^{32}P]$ Ptd OH levels when compared to either saline (120s) or to the inactive

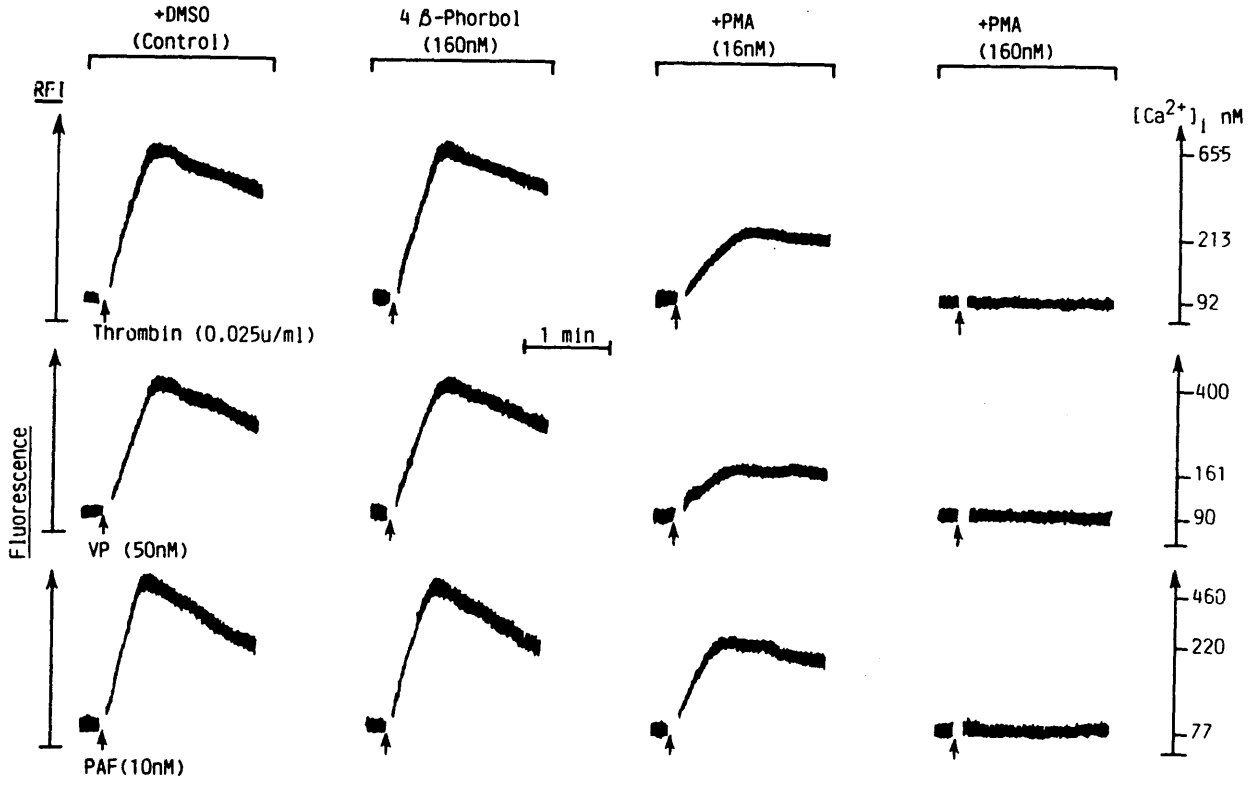

Figure 45: Effect of PMA on agonist-induced elevation of $[Ca^{2+}]_i$.

2.0 ml of Quin 2 labelled platelets were pre-incubated for 120s with DMSO (vehicle), 4 β phorbol or PMA at the concentrations indicated. Fluorescence responses were monitored following the addition of thrombin (0.025U/ml (1.25 nM)), vasopressin (50 nM) or PAF (10 nM) and the appropriate calibration for $[Ca^{2+}]_i$ is shown to the right of the fluorescence records. The experiment shown is typical of 3 similar experiments.

phorbol analogue, 4βphorbol (1.6 μM; 120s). In contrast PMA (1.6 μM; 120s) did cause a significant decrease in the levels of $[^{32}P]$ Ptd Ins (Figures 35 & 46).

Figure 47 depicts the effect of PMA on PAF induced $[^{32}P]$ Ptd OH formation. In this experiment PAF (10 nM; 30s) elicited a 523 ± 11% response following preincubation with 4βphorbol (1.6 μM; 120s). This was attenuated, in a concentration dependent manner, by preincubation with PMA (16 nM - 1.6 μM; 120s). Moreover PMA preincubation (100 nM; 120s) suppressed the response to PAF at all concentrations of the agonist tested (1 - 100 nM; 30s) (Figure 48). Preincubation with PMA (16 nM - 1.6 μM; 120s) also attenuated thrombin (0.1U/ml (5nM); 30s) induced $[^{32}P]$ Ptd OH formation in a concentration dependent manner (Figure 49) and 100 nM PMA for 120s caused an apparent rightward shift in the thrombin concentration response curve (Figure 50). Vasopressin (100 nM; 30s) induced 338 ± 48% of the control level of $[^{32}P]$ Ptd OH following preincubation with 4βphorbol (1.6 μM; 120s). This stimulation was inhibited in a concentration dependent manner by pre-incubation with PMA (16 nM - 1.6 μM; 120s) as seen in Figure 51. EP 171 (10 nM; 120s) elicited an increase to 257 ± 24% of the basal $[^{32}P]$ Ptd OH levels. This was not significantly altered by 120s preincubation with DMSO (228 ± 10% of control). In contrast the addition of PMA (100 nM; 120s) prior to challenge with EP 171 (10 nM; 120s) truncated the formation of $[^{32}P]$ Ptd OH to 126 ± 7% of control (Figure 52). In addition

PMA (100 nM; 120s) markedly suppressed the response (Ptd OH formation) elicited by all concentrations of EP 171 tested (Figure 53). Similar effects were observed when platelets were preincubated with PDBu (200 nM; 120s) prior to challenge with PAF (10 nM; 30s), thrombin (0.1U/ml (5 nM); 30s) or vasopressin (100 nM; 30s) (Figure 54).

To examine the effects of protein kinase C activation on agonist-induced poly-phosphoinositide metabolism thrombin was used as it is the most efficacious agonist available. PMA (100 nM; 120s) induced a significant increase in $[^{32}P]$ -PIP_2 and -PIP levels (127 $\pm$ 1% and 177 $\pm$ 4% of DMSO control respectively). Thrombin (1U/ml (50 nM); 10s) caused a decrease in PIP_2 (86 $\pm$ 4% of saline control) but did not alter PIP levels (101 $\pm$ 2%). Preincubation with PMA (100 nM; 120s) inhibited the thrombin induced decrease in $[^{32}P]$ PIP_2 levels to 123 $\pm$ 3% which was not significantly different from PMA alone (127 $\pm$ 1%) (Figure 55).

Therefore phorbol esters presumably by activation of protein kinase C have a pronounced effect on the human platelet phosphoinositide cycle. PMA decreases $[^{32}P]$ Ptd Ins levels, increases both $[^{32}P]$ PIP_2 and $[^{32}P]$ PIP (PIP > PIP_2) and apparently inhibits the thrombin induced hydrolysis of PIP_2. In addition PMA inhibits agonist-induced formation of $[^{32}P]$ Ptd OH with no effect on the basal Ptd OH levels. As phorbol esters effectively act as DAG mimetics, it is possible that DAG (via activation of protein kinase C) plays not only a stimulatory role but also an inhibitory (negative feedback)

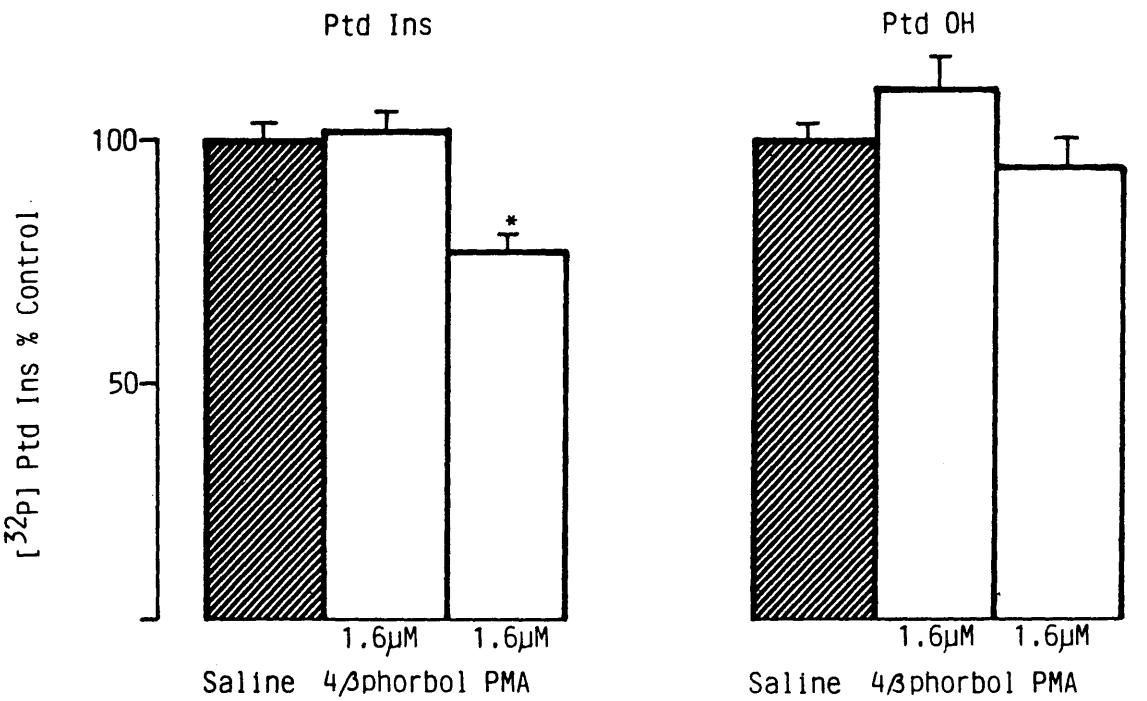

Figure 46. Effect of PMA on $[^{32}P]$ Ptd OH and $[^{32}P]$ Ptd Ins levels.

0.4 ml samples of $[^{32}P]$ P_i prelabelled platelets were exposed to either saline, 4$^{\beta}$phorbol or PMA at the concentrations indicated. $[^{32}P]$ Ptd OH and $[^{32}P]$ Ptd Ins were measured 120s after agonist addition. The results are means $\pm$ SEM of triplicate determinations. The experiment is typical of 8 similar experiments. Analysis of data: *$p < 0.005$.

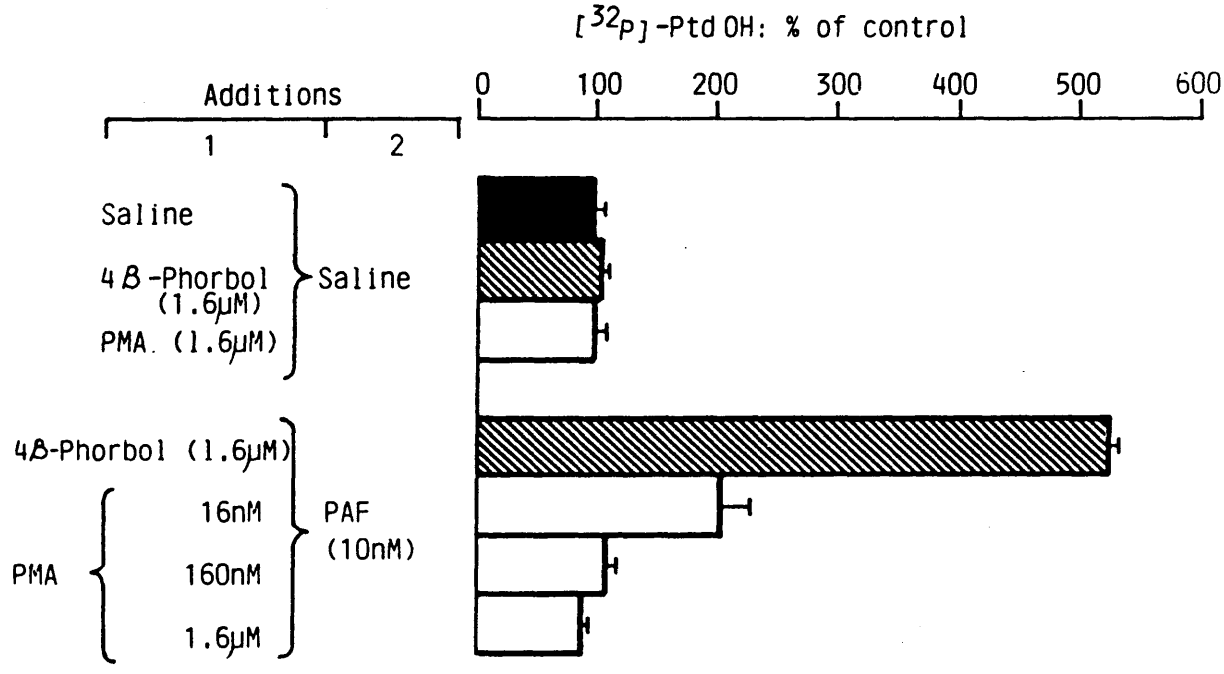

Figure 47. Effect of PMA on PAF-induced stimulation of $[^{32}P]$ Ptd OH formation.

0.4 ml samples of $[^{32}P]$ P_i prelabelled platelets were pre-incubated for 120s with either saline (closed bars) 4 βphorbol (hatched bars) or PMA (open bars) at the concentration indicated. Reactions were initiated by the addition of either saline (control) or 10 nM PAF. $[^{32}P]$ Ptd OH levels were measured 30s after agonist addition. The results are means ± SEM of triplicate determinations. The experiment is typical of 2 similar experiments.

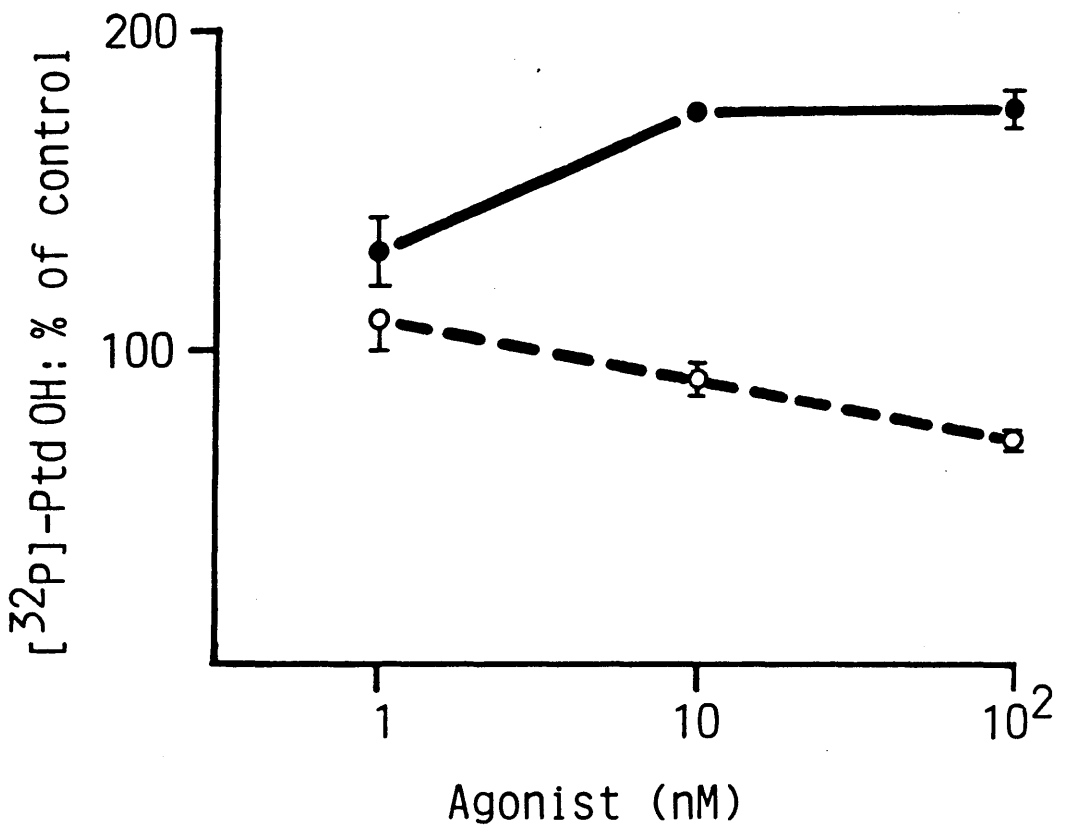

Figure 48. Effect of PMA on concentration response curve for PAF-induced stimulation of $[^{32}P]$ Ptd OH formation.

0.4 ml samples of $[^{32}P]$ P_i prelabelled platelets were pre-incubated for 120s with either DMSO (closed circles) or 100 nM PMA (open circles). Reactions were initiated by the addition of PAF at the concentrations indicated. $[^{32}P]$ Ptd OH levels were measured 30s after agonist addition. The results are means $\pm$ SEM of triplicate determinations. The experiment is typical of 2 similar experiments.

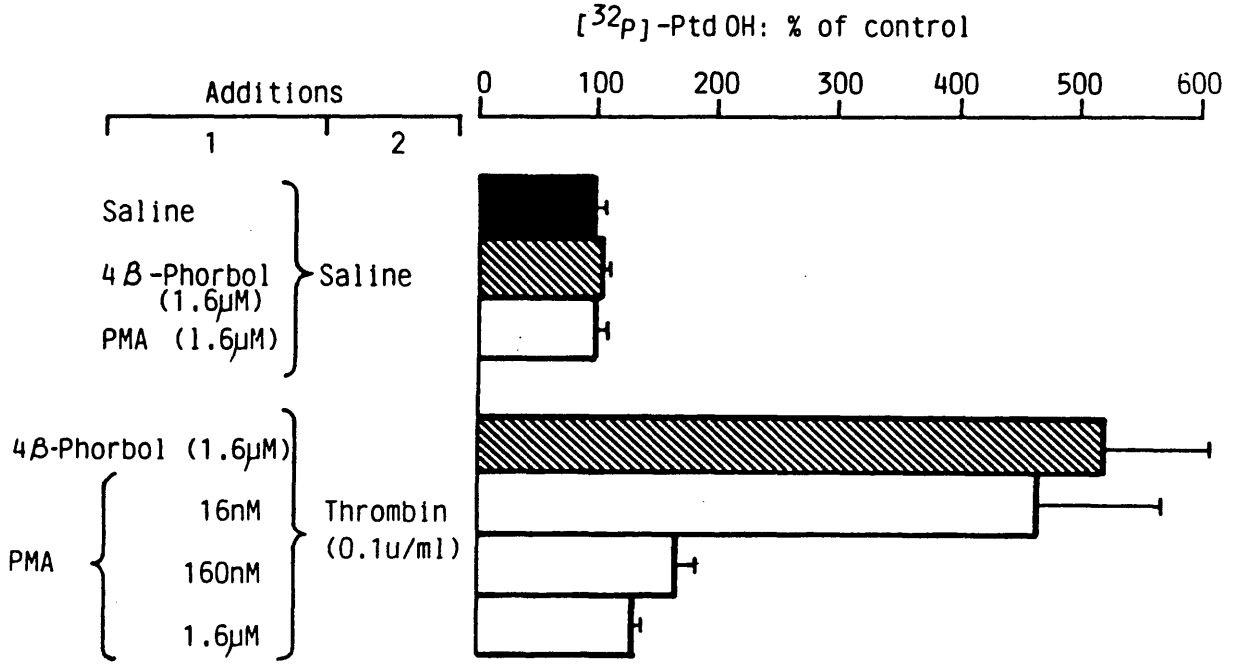

Figure 49. Effect of PMA on thrombin-induced stimulation of $[^{32}P]$ Ptd OH formation.

0.4 ml samples of $[^{32}P]$ P_i prelabelled platelets were pre-incubated for 120s with either saline (closed bars), 4 β phorbol (hatched bars) or PMA (open bars) at the concentrations indicated. Reactions were initiated by the addition of either saline (control) or 0.01U/ml (5 nM) thrombin. $[^{32}P]$ Ptd OH levels were measured 30s after agonist addition. The results are means ± SEM of triplicate determinations. The experiment is typical of 2 similar experiments.

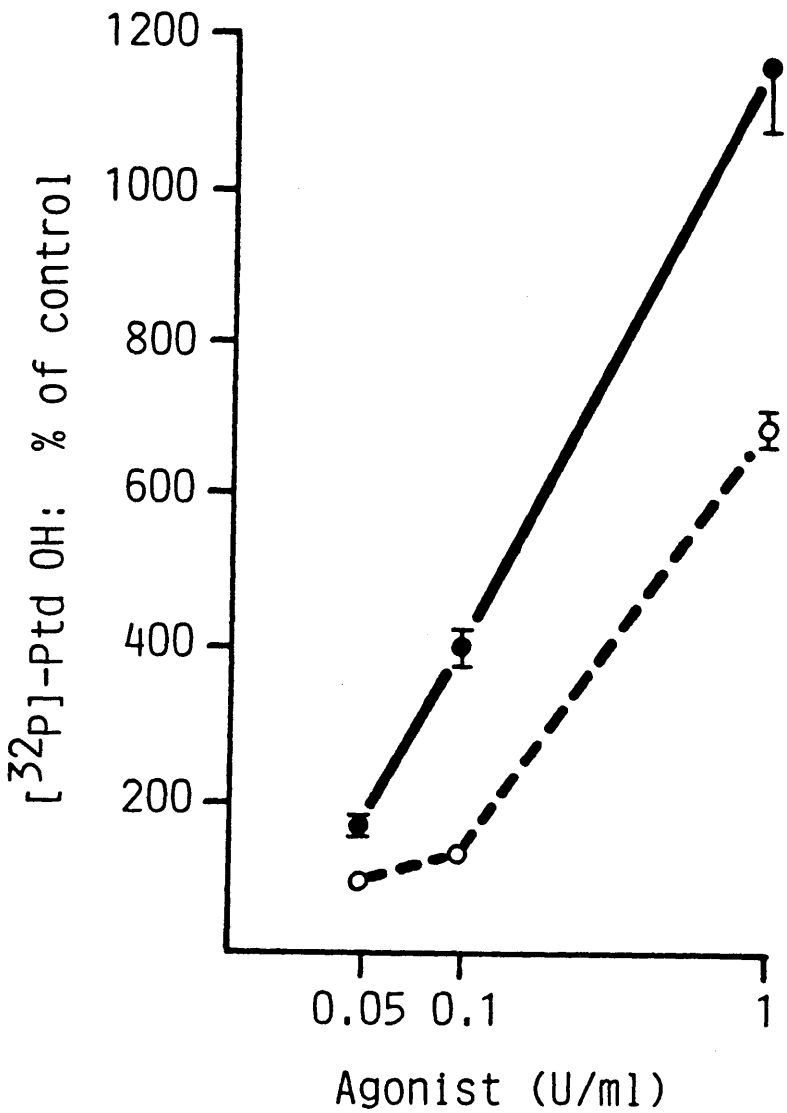

Figure 50. Effect of PMA on the concentration response curve for thrombin-induced stimulation of $[^{32}P]$ Ptd OH formation.

0.4 ml samples of $[^{32}P]$ P_i prelabelled platelets were incubated for 120s with either DMSO (closed circles) or 100 nM PMA (open circles). Reactions were initiated by the addition of thrombin at the concentrations indicated. $[^{32}P]$ Ptd OH levels were measured 30s after agonist addition. The results are means ± SEM of triplicate determinations. The experiment is typical of 2 similar experiments.

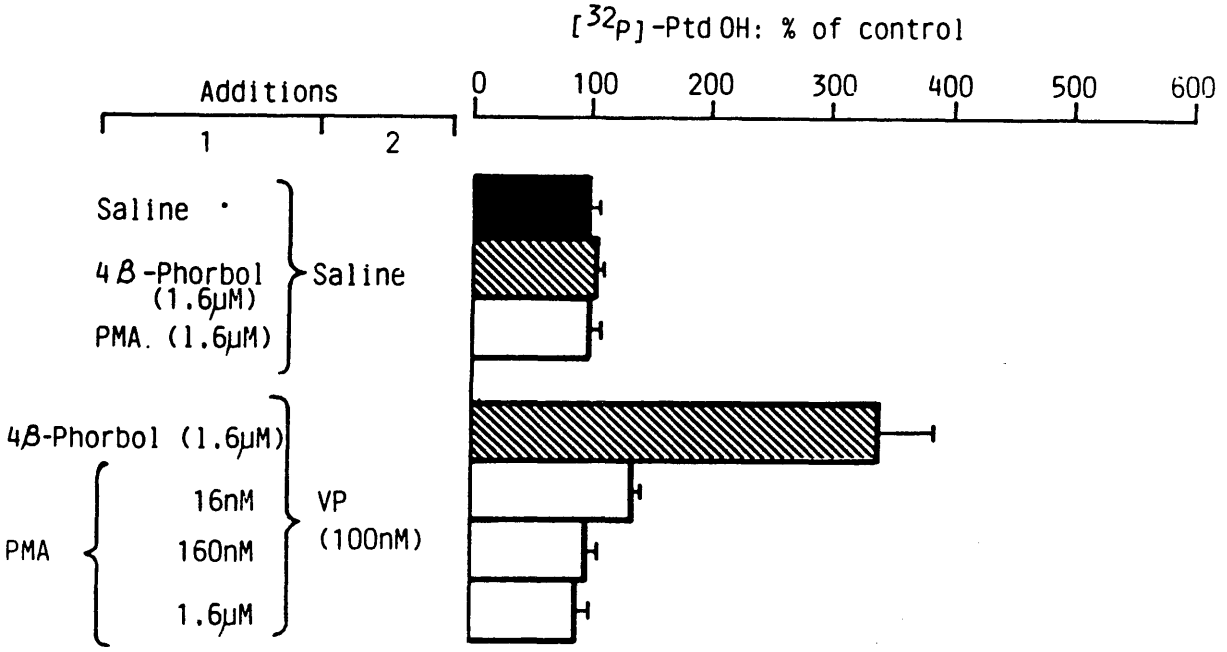

Figure 51. Effect of PMA on vasopressin-induced stimulation of $[^{32}P]$ Ptd OH formation.

0.4 ml samples of $[^{32}P]$ P_i prelabelled platelets were pre-incubated for 120s with either saline (closed bars), 4β phorbol (hatched bars) or PMA (open bars) at the concentrations indicated. Reactions were initiated by the addition of either saline or 100 nM vasopressin. $[^{32}P]$ Ptd OH levels were measured 30s after agonist addition. The results are means ± SEM of triplicate determinations. The experiment is typical of 2 similar experiments.

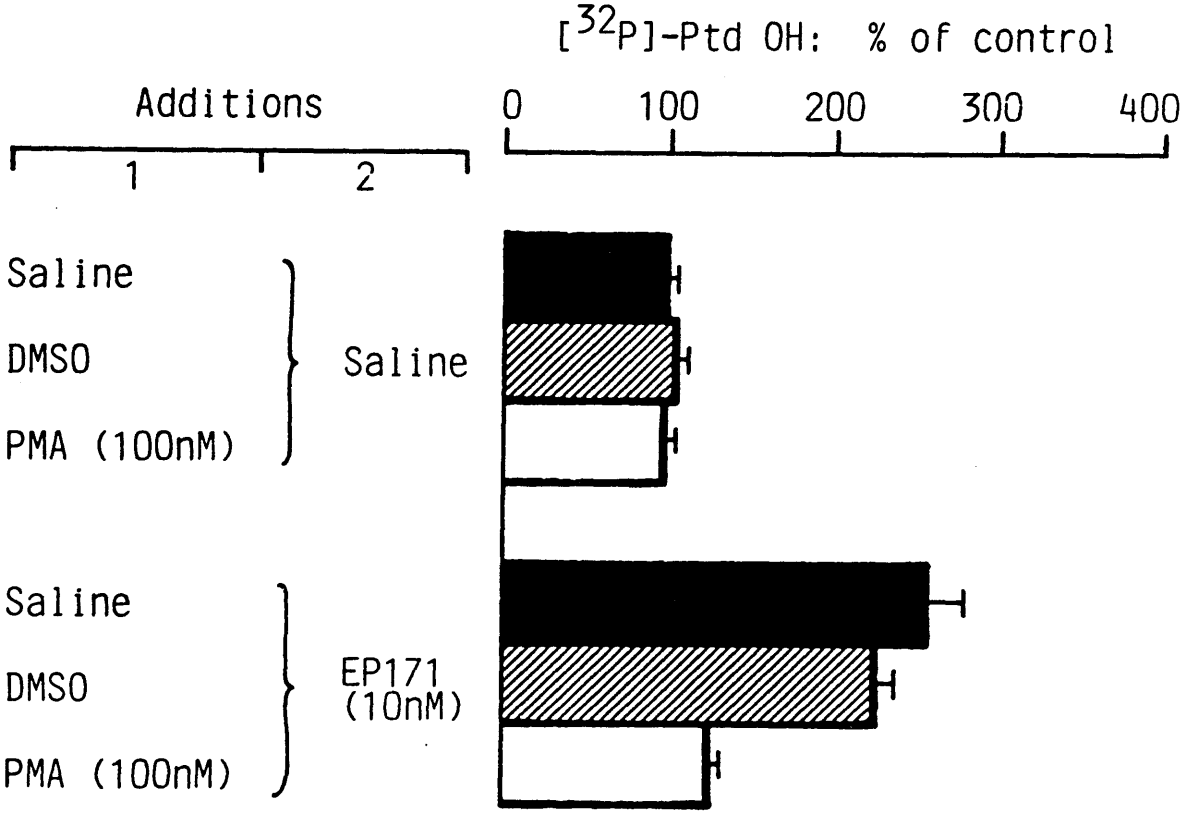

Figure 52. Effect of PMA on EP 171-induced stimulation of $[^{32}P]$ Ptd OH formation.

0.4 ml samples of $[^{32}P]$ P_i prelabelled platelets were pre-incubated for 120s with either saline (closed bars), DMSO (hatched bars) or 100 nM PMA (open bars). Reactions were initiated by the addition of either saline or 10 nM EP 171. $[^{32}P]$ Ptd OH levels were measured 120s after agonist addition. The results are means $\pm$ SEM of triplicate determinations. The experiment is typical of 2 similar experiments.

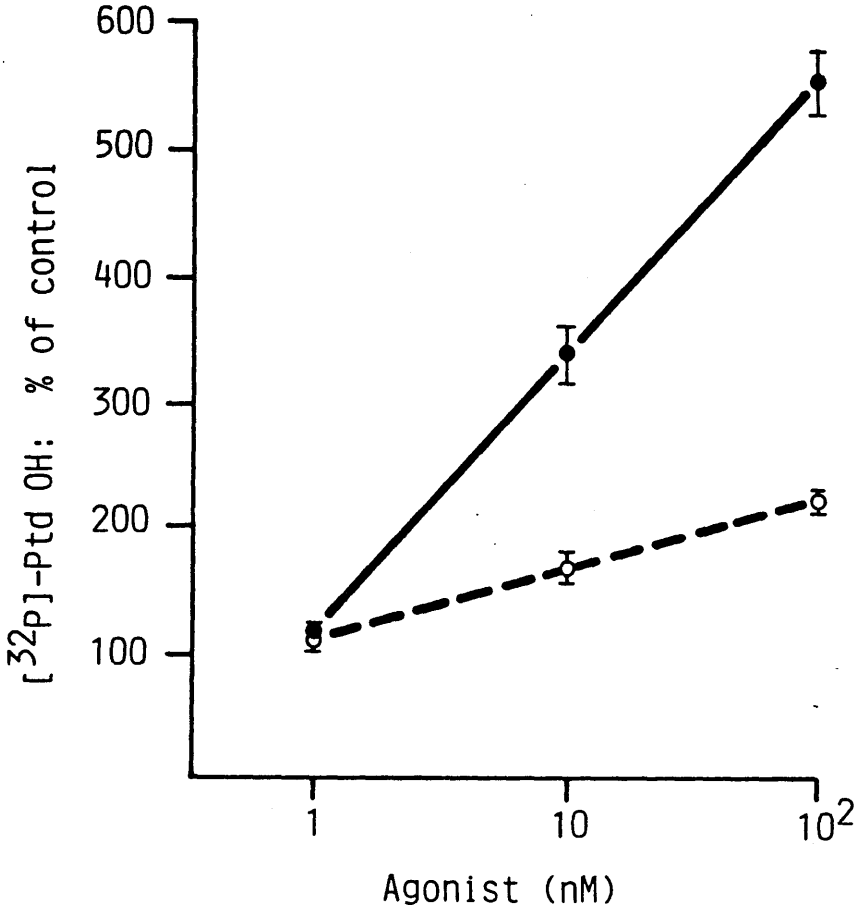

Figure 53. Effects of PMA on the concentration response curve for EP 171-stimulation of $[^{32}P]$ Ptd OH formation.

0.4 ml samples of $[^{32}P]$ P_i prelabelled platelets were pre-incubated with either DMSO (closed circles) or 100 nM PMA (open circles). Reactions were initiated by the addition of EP 171 at the concentration indicated. $[^{32}P]$ Ptd OH levels were measured 120s after agonist addition. The results are means $\pm$ SEM of triplicate determinations. The experiment is typical of 2 similar experiments.

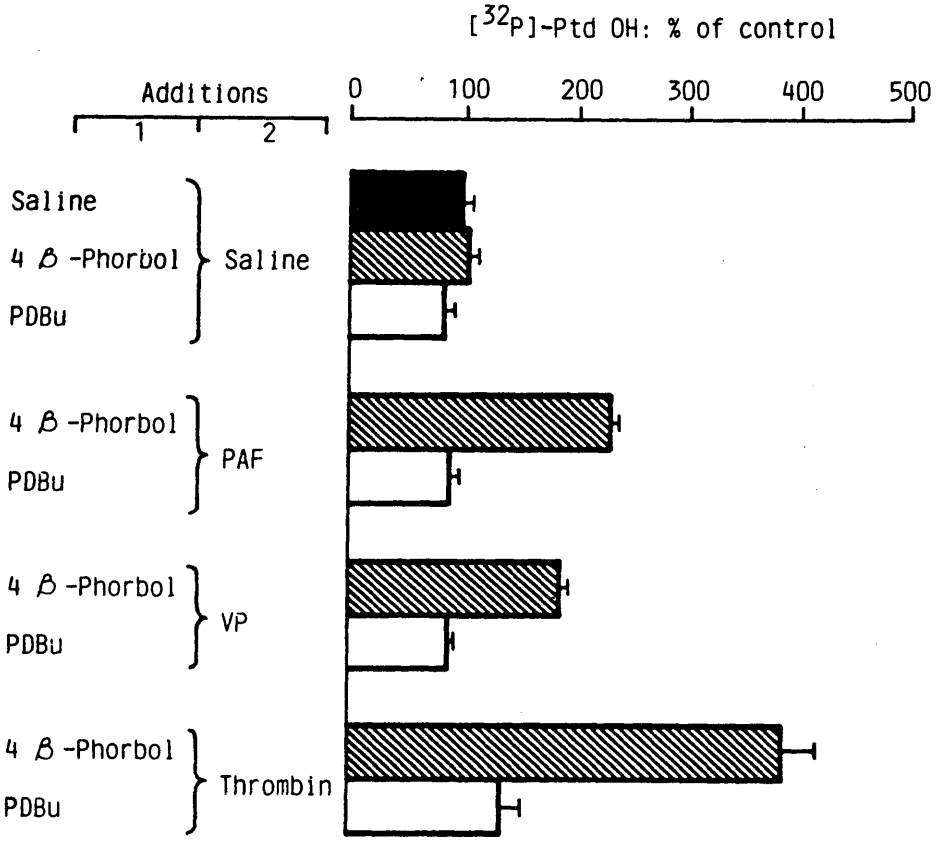

Figure 54. Effects of PDBu on agonist-induced stimulation of $[^{32}P]$ Ptd OH formation.

0.4 ml samples of $[^{32}P]$ P_i prelabelled platelets were pre-incubated for 120s with either saline (closed bar), 4 β phorbol (hatched bars) or 200 nM PDBu (open bars). Reactions were initiated by the addition of either saline, 10 nM PAF or 100 nM vasopressin or 0.1U/ml (5 nM) thrombin. $[^{32}P]$ Ptd OH levels were measured 30s after agonist addition. The results are means ± SEM of triplicate determinations. The experiment is typical of 3 similar experiments.

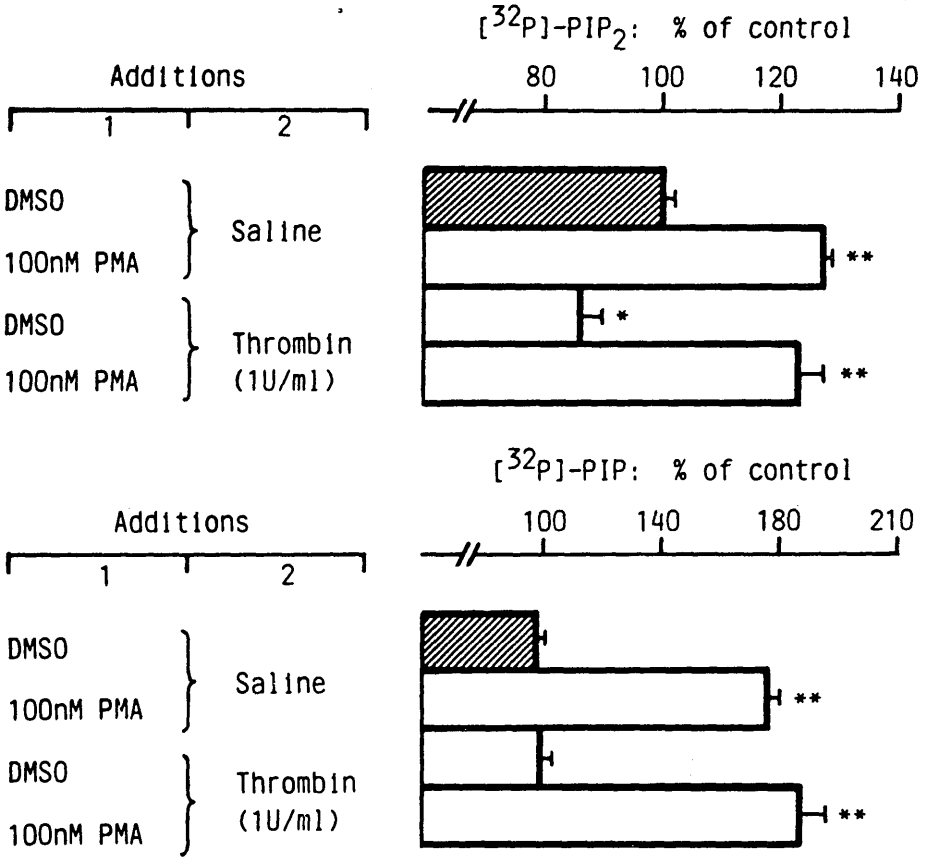

Figure 55. Effects of PMA on thrombin-induced changes in levels of $[^{32}P]$ poly phosphoinositides.

0.4 ml samples of $[^{32}P]$ P_i pulse chase labelled platelets were pre-incubated for 120s with either DMSO or 100 nM PMA as depicted above. Reactions were initiated with either saline or 1U/ml (50 nM) thrombin and the levels of $[^{32}P]$ PIP_2 (upper trace) and $[^{32}P]$ PIP (lower trace) were measured 10s after agonist addition. The results are means ± SEM of triplicate determinations. The experiment is typical of 2 similar experiments.

role on the transduction processes that are initiated by occupancy of platelet receptors for stimulatory agonists.

Having established that prior exposure of platelets to agents that elevate cAMP, cGMP or activate protein kinase C can impair transduction processes activated by subsequent challenge with agonists, I next investigated whether the same agents could reverse the transduction process once initiated.

2.8 Reversal of agonist induced elevation of platelet $[Ca^{2+}]_i$

The possible role of endogenous cyclic nucleotides and DAG in mediating the reversal of agonist induced elevation of $[Ca^{2+}]_i$ was investigated. A sub-maximal PAF concentration (50 nM) elicits an increase in fluorescence in Quin 2 labelled platelets which is followed by a decline in the signal. As depicted in Figure 56, PGI_2 (10 nM - 1 μM), NaNP (1 - 10 μM) and PMA (50 - 100 nM) added at the peak of the fluorescence signal all accelerated, in a concentration dependent manner, the rate of this decline. This suggests that cAMP, cGMP and DAG may play a role in limiting or terminating the agonist induced response.

2.9 Reversal of agonist induced platelet phosphoinositide metabolism

To further investigate the ability of cAMP, cGMP and DAG to modulate the platelet transduction process, the ability of agents which influence their activity to terminate agonist induced phosphoinositide hydrolysis was examined. The agonist used in the previous studies, PAF, was not considered appropriate

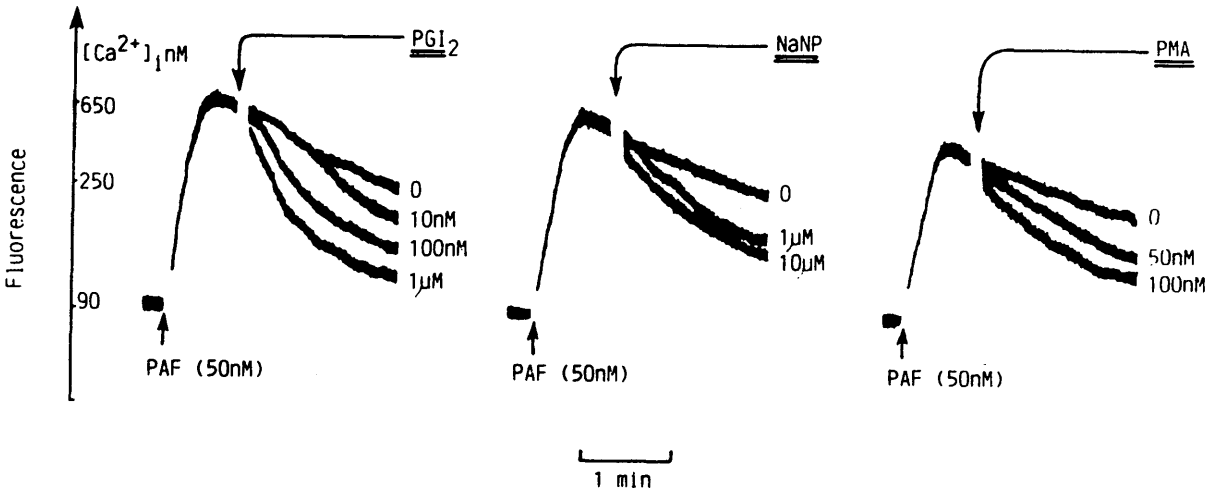

Figure 56. Reversal of PAF-induced elevation of $[Ca^{2+}]_i$ by PGI_2, NaNP and PMA.

2.0 ml of Quin 2 labelled platelets were challenged with 50 nM PAF, PGI_2, NaNP or PMA, at the concentrations indicated, or the appropriate vehicle was added at the peak of the resultant fluorescence signal and the $[Ca^{2+}]_i$ calibrated from the observed changes. The experiment shown is typical of at least 4 experiments for each agent.

in this section. This was because an agonist was required which, at sub-maximal concentrations, stimulates $[^{32}P]$ Ptd OH formation to a prolonged elevated level which is large enough to observe the possible inhibitory effects of NaNP, PGD_2 and PMA. The formation of $[^{32}P]$ Ptd OH by 18 nM PAF, although well maintained, would be insufficient to monitor inhibitory actions (Figure 38).

A sub-maximal EP 171 concentration (10 nM) stimulated the formation of $[^{32}P]$ Ptd OH which reached a peak at 120s and remained at this elevated level at 600s (Figure 57). The profile of this curve is similar to that of the submaximal PAF concentration (Figure 38). The curve was prolonged at a level which allowed the effect of the subsequent addition of a compound to be observed.

The addition of PGD_2 (100 nM - 1 μM) or NaNP (100 nM - 1 μM) 30s after EP 171 (10 nM) suppressed the Ptd OH formation (when compared to the saline controls) monitored 60s later (Figures 58 & 59). At the concentrations used, PGD_2 stimulates both adenylate and guanylate cyclases whereas NaNP specifically elevates cGMP levels (MacIntyre *et al.*, 1985a). Thus this result does not differentiate between the effects of PGD_2 on Ptd OH formation being mediated by cAMP and/or cGMP. This point was clarified by examining the effects of the stable cyclic nucleotide analogues 8 Br cAMP and 8 Br cGMP on EP 171 elevated levels of $[^{32}P]$ Ptd OH. In this experiment the extent of $[^{32}P]$ Ptd OH formation induced by EP 171 (10 nM) within

30s was 263 ± 41% of control. This increased to 760 ± 13% of pre-stimulus level after the addition of saline for a further 300s (i.e. 330s after EP 171). 8 Br cAMP (1 mM) added 30s after EP 171 had no effect (743 ± 16%) on the level of Ptd OH observed at 330s, however it was significantly reduced to 423 ± 56% by the addition at 30s of 8 Br cGMP (1 mM) for a further 300s (Figure 60). This suggests that cGMP is more effective than cAMP at reversing agonist induced formation of $[^{32}P]$ Ptd OH. It is possible that the PGD_2-induced reversal of Ptd OH formation is mediated by a non-specific stimulation of guanylate cyclase and not due to its activation of adenylate cyclase. The effect of PMA on elevated $[^{32}P]$ Ptd OH levels induced by EP 171 is shown in Figure 61. EP 171 (10 nM) for 30s elevated $[^{32}P]$ Ptd OH to 466 ± 6% of the pre-stimulus control. PMA (100 nM - 1 μM) or DMSO the vehicle control were added 30s after EP 171 and the levels of $[^{32}P]$ Ptd OH were monitored 60s later. Both concentrations of PMA reduced the Ptd OH (to 246 ± 18% & 226 ± 18% respectively of the pre-stimulus level) levels to below that of DMSO (604 ± 10%).

Hence PGD_2, NaNP and PMA, all of which inhibit agonist induced elevation of $[Ca^{2+}]_i$ and $[^{32}P]$ Ptd OH formation and reverse an agonist evoked calcium signal, also all reverse an agonist stimulated $[^{32}P]$ Ptd OH levels.

2.10 Effect of PMA on thrombin induced arachidonate release

Having established that phosphoinositide hydrolysis and Ca^{2+} flux could be attenuated by agents that activate protein kinase C, I next examined the effects of activation of protein kinase C

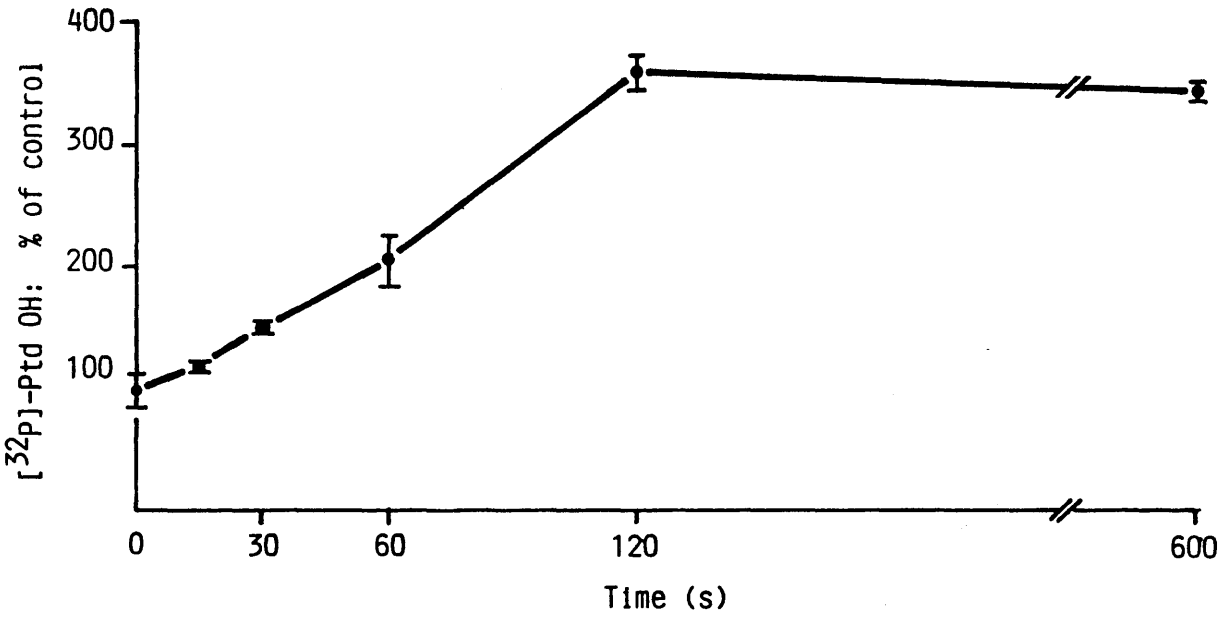

Figure 57. Time course of low EP 171 concentration-induced stimulation of $[^{32}P]$ Ptd OH formation.

0.4 ml samples of $[^{32}P]$ P_i prelabelled platelets were exposed to 10 nM EP 171 and the levels of $[^{32}P]$ Ptd OH measured at the times indicated. The results are means ± SEM of triplicate determinations. The experiment is typical of 2 similar experiments.

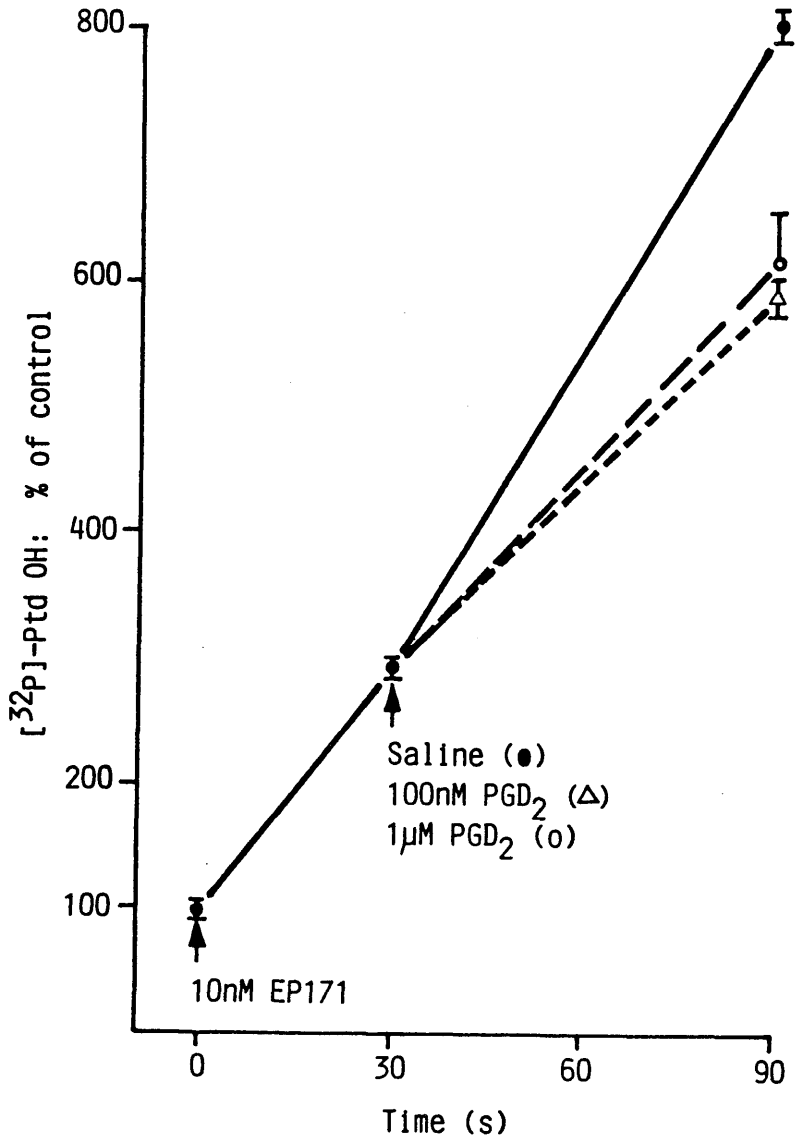

Figure 58. Reversal of EP 171-induced stimulation of $[^{32}P]$ Ptd OH formation by PGD_2.

0.4 ml samples of $[^{32}P]$ P_i prelabelled platelets were exposed to 10 nM EP 171 and 30s after agonist addition either saline (closed circle), 100 nM (open triangle) or 1 μ M (open circle) PGD_2 was added. The levels of $[^{32}P]$ Ptd OH were measured at the times indicated. The results are means ± SEM of triplicate determinations. The experiment is typical of 2 similar experiments.

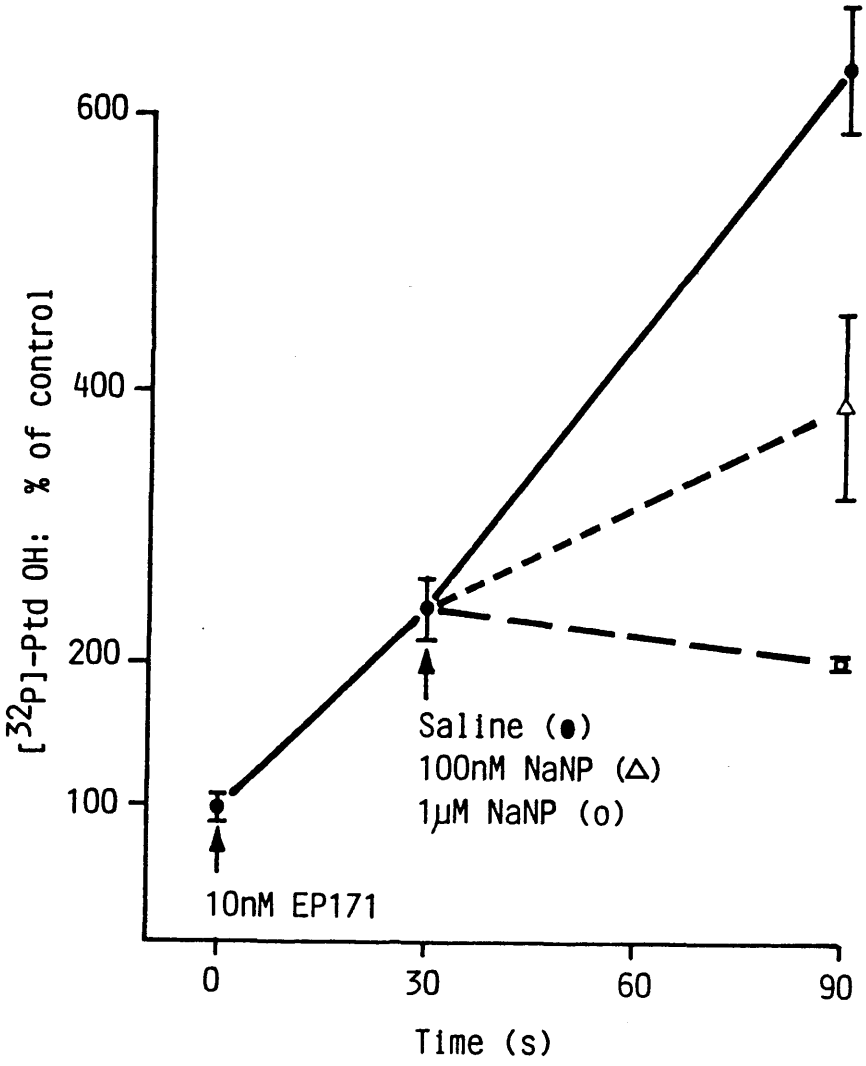

Figure 59. Reversal of EP 171-induced stimulation of $[^{32}P]$ Ptd OH formation by NaNP.

0.4 ml samples of $[^{32}P]$ P_i prelabelled platelets were exposed to 10 nM EP 171 and 30s after agonist addition either saline (closed circle), 100 nM (open triangle) or 1 μM (open circle) NaNP was added. The levels of $[^{32}P]$ Ptd OH were measured at the times indicated. The results are means ± SEM of triplicate determinations. The experiment is typical of 2 similar experiments.

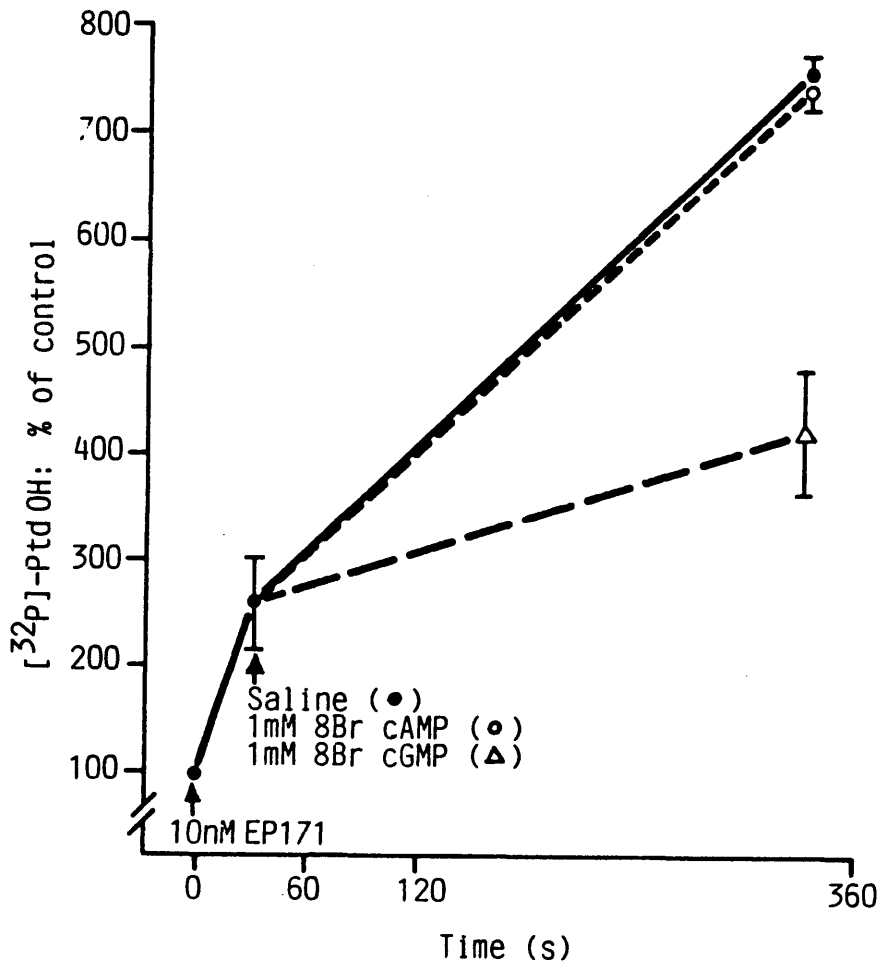

Figure 60. Reversal of EP 171-induced stimulation of $[^{32}P]$ Ptd OH formation by 8 Br cAMP and 8 Br cGMP.

0.4 ml samples of $[^{32}P]$ P_i prelabelled platelets were exposed to 10 nM EP 171 and 30s after agonist addition either saline (closed circle), 1 mM 8 Br cAMP (open circle) or 1 mM 8 Br cGMP (open triangle) was added. The levels of $[^{32}P]$ Ptd OH were measured at the times indicated. The results are means ± SEM of triplicate determinations. The experiment is typical of 2 similar experiments.

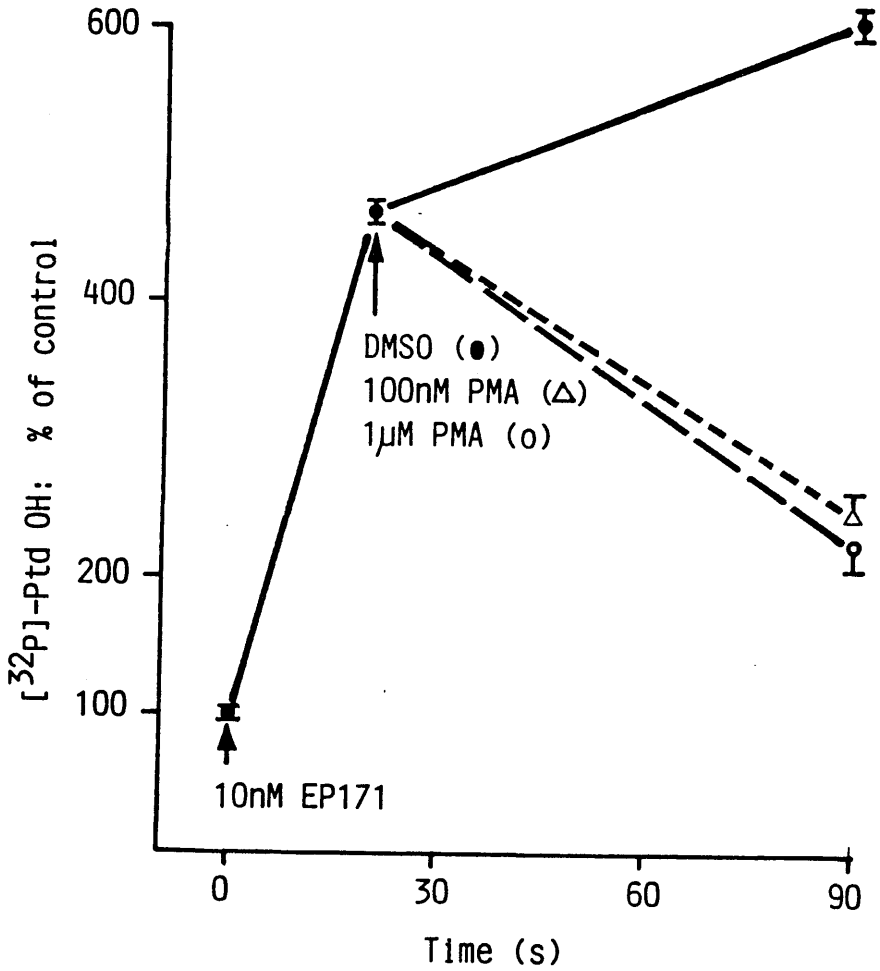

Figure 61. Reversal of EP 171-induced stimulation of $[^{32}P]$ Ptd OH formation by PMA.

0.4 ml samples of $[^{32}P]$ P_i prelabelled platelets were exposed to 10 nM EP 171 and 30s after agonist addition either DMSO (closed circle), 100 nM (open triangle) or 1 μM (open circle) PMA was added. The levels of $[^{32}P]$ Ptd OH were measured at the times indicated. The results are means ± SEM of triplicate determinations. The experiment is typical of 2 similar experiments.

on the liberation of arachidonic acid. This liberation of arachidonic acid from phospholipids is believed to be mediated by a Ca^{2+}-activated acyl-hydrolase (phospholipase A_2) (Wong & Cheung, 1979).

PMA (100 nM - 1 μM; 120s) did not cause the release of $[^3H]$ arachidonate when compared to the DMSO control. Thrombin (1U/ml (50 nM); 120s) stimulated release of $[^3H]$ arachidonate which was unaffected by preincubation of PMA (100 nM - 1 μM; 120s) (Figure 62). It appears therefore that although PMA has pronounced effects on the platelet transduction process it does not alter agonist induced arachidonate release.

2.11 Summary

The observation that increasing agonist concentration accelerates the decline in levels of stimulatory second messengers (either by impairing formation or stimulating catabolism) is perhaps indicative of some negative feedback system in the platelet. The most obvious endogenous candidates to mediate this feedback would be cAMP, cGMP and DAG. Agents which mimic, or stimulate the formation of each of these compounds inhibit the formation of, and enhance the rate of decline of, both $[Ca^{2+}]_i$ and $[^{32}P]$ Ptd OH. As cGMP and DAG reportedly are formed in platelets challenged with agonists, these findings suggest that either of these may play a role as endogenous agents which limit or terminate agonist induced platelet activation.

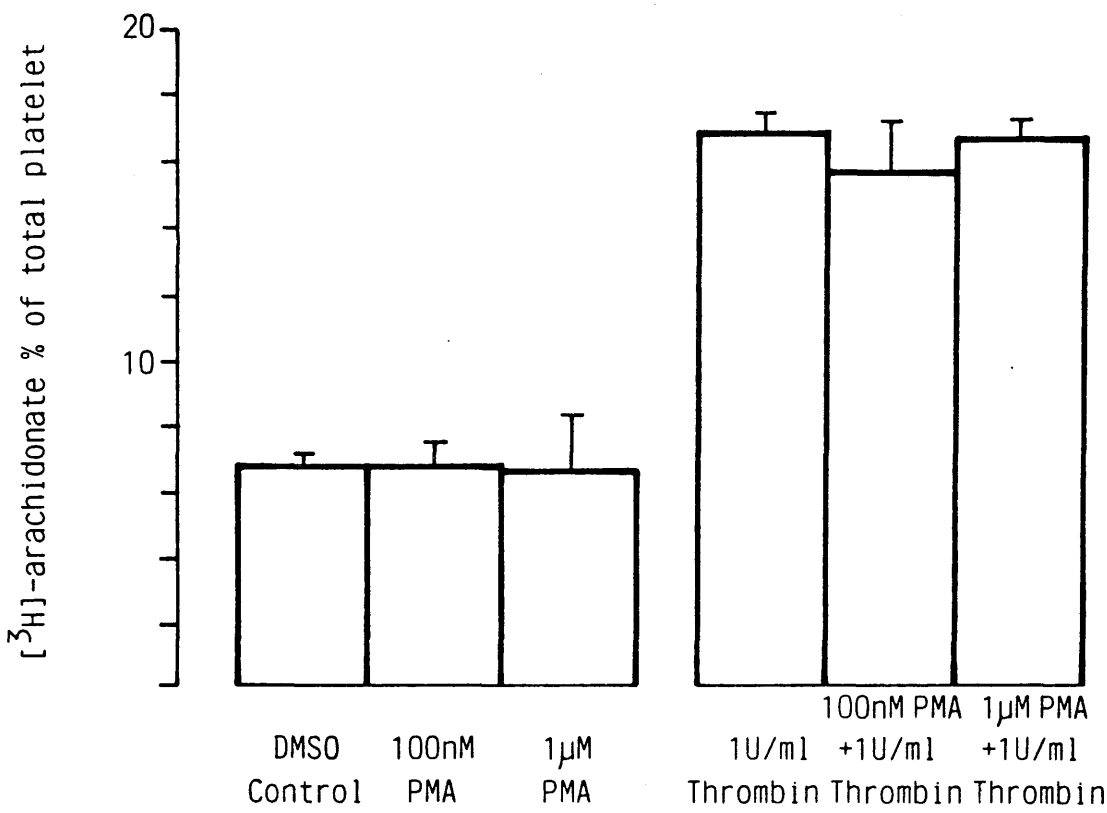

Figure 62. Effects of PMA on thrombin-induced release of $[^3H]$ arachidonate.

0.2 ml of $[^3H]$ arachidonic acid prelabelled platelets were pre-incubated for 120s with either DMSO or PMA at the concentrations indicated. Thrombin (1.0U/ml (50 nM) was subsequently added for 120s and the $[^3H]$ arachidonate release monitored. The results are means $\pm$ SEM of triplicate determinations. The experiment is typical of 7 similar experiments.

3. Mechanisms underlying potentiation

ADP, but not adrenaline, induces an increase in $[Ca^{2+}]_i$ (MacIntyre et al., 1985b), however neither stimulate $[^{32}P]$ Ptd OH formation (Figures 18, 19 & 20; MacIntyre et al., 1985b). Both of these agents are capable of potentiating the platelet functional response evoked by other agonists. The mechanism underlying this effect is unclear, although both ADP and adrenaline are linked to the inhibition of adenylate cyclase, suggesting perhaps some function for decreased cAMP levels in this phenomenon. The possibility of an augmentation of phosphoinositide metabolism (possibly via inhibition of adenylate cyclase) mediating this potentiation has not been investigated. Consequently I investigated the effects of adrenaline and ADP on agonist induced phosphoinositide hydrolysis.

3.1 Effect of ADP on agonist induced platelet phosphoinositide metabolism

ADP per se does not stimulate Ptd OH formation (Figures 18 & 19). However, when added to platelets pre-incubated with flurbiprofen (10 μM), ADP (10 μM; 60s) resulted in a shift in the concentration response curves for $[^{32}P]$ Ptd OH stimulated by PAF (1.8 - 180 nM; 30s) and by vasopressin (3 - 300 nM; 30s). The maximum responses were increased from 213 ± 13% to 258 ± 18% and from 198 ± 7% to 253 ± 7% respectively (Figures 63 & 64). The presence of flurbiprofen indicates that this potentiation is not dependent on platelet cyclo oxygenase activity, and thus cannot be mediated by endogenous eicosanoids (PGG_2, PGH_2, TxA_2).

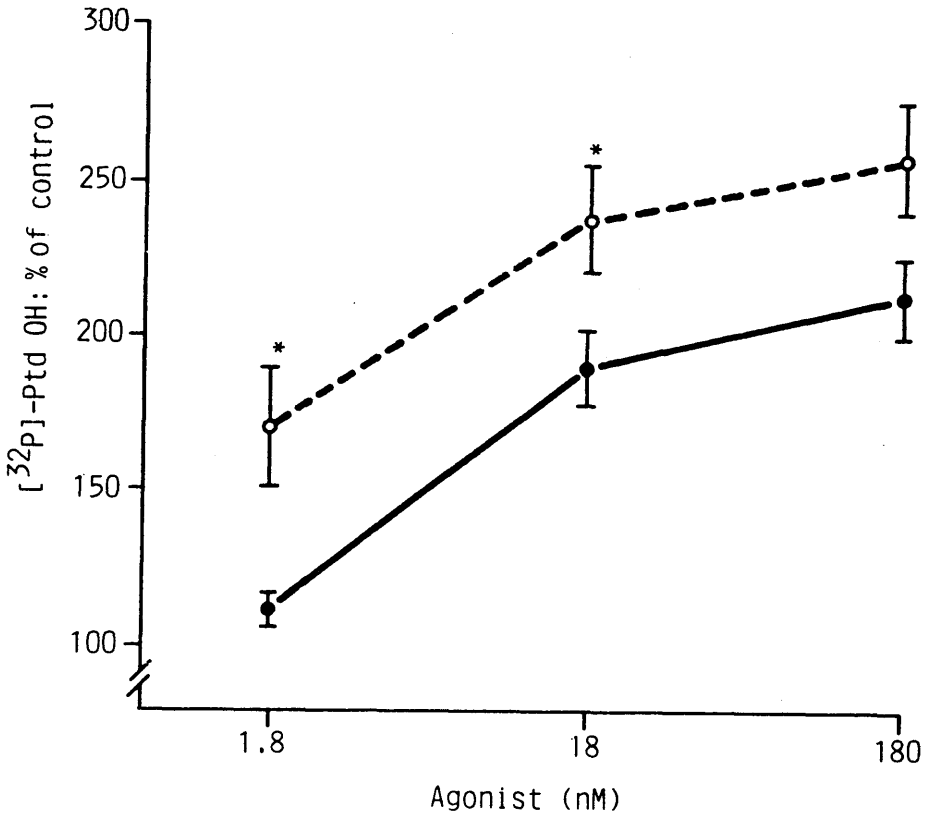

Figure 63. Effect of ADP on the concentration response ourve for PAF-induced stimulation of $[^{32}P]$ Ptd OH formation.

0.4 ml samples of $[^{32}P]$ P_i prelabelled, flurbiprofen pretreated platelets were pre-incubated for 60s with either saline (closed circles) or 10 μM ADP (open circles), prior to the addition of PAF at the concentrations indicated. $[^{32}P]$ Ptd OH levels were measured 30s after PAF addition. The results are means ± SEM of 3 experiments each with triplicate determinations. Analysis of data: *$p < 0.05$.

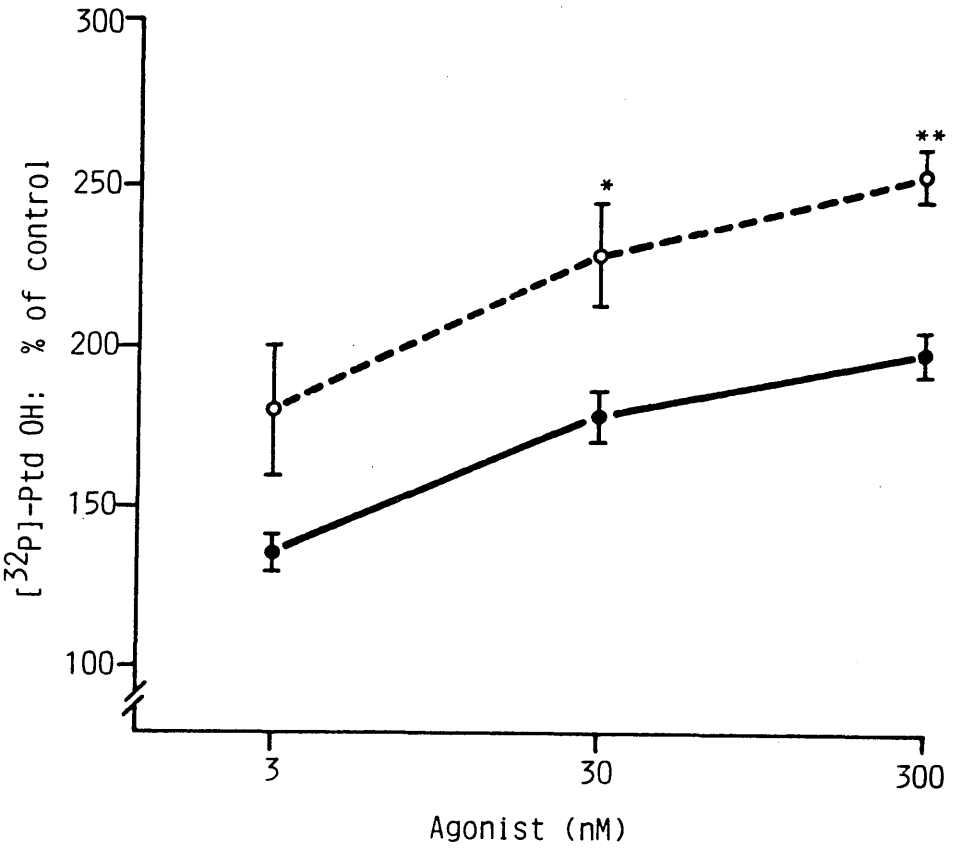

Figure 64. Effect of ADP on the concentration response curve for vasopressin-induced stimulation of $[^{32}P]$ Ptd OH formation.

0.4 ml samples of $[^{32}P]$ P_i prelabelled, flurbiprofen pretreated platelets were pre-incubated for 60s with either saline (closed circles) or 10 μM ADP (open circles), prior to the addition of vasopressin at the concentrations indicated. $[^{32}P]$ Ptd OH levels were measured 30s after vasopressin addition. The results are means ± SEM of 3 experiments each with triplicate determinations. Analysis of data: *$p < 0.01$; **$p < 0.005$.

3.2 Effect of adrenaline on agonist induced platelet phosphoinositide metabolism

The effects of adrenaline on PAF, vasopressin and thrombin induced formation of $[^{32}P]$ Ptd OH was investigated in platelets pretreated with flurbiprofen (10 μM). PAF (1.8 - 180 nM; 30s) induced a concentration dependent formation of Ptd OH (Figure 65). Adrenaline (5 μM; 60s) preincubation caused a significant potentiation of the sub-maximal PAF responses (1.8 - 18 nM) but not of the maximum PAF concentration (180 nM). Similarly, adrenaline potentiated thrombin (0.01 - 1U/ml (0.5 - 50 nM); 30s) induced Ptd OH formation when elicited by low (0.01 - 0.1U/ml) but not by high thrombin concentrations (Figure 66). In identical conditions adrenaline potentiated vasopressin (10 - 300 nM; 30s) induced Ptd OH formation at all concentrations (Figure 67).

ADP fails to evoke phosphoinositide metabolism monitored by changes in Ptd OH (Figures 18 & 19), PIP or PIP_2 (Figure 20) in $[^{32}P]$ P_i prelabelled platelets. It is possible that these assays are not sufficiently sensitive to detect ADP induced phosphoinosiitide hydrolysis. Were this to be the case, adrenaline might potentiate the response to a detectable level. Figure 68 depicts the (non) effects of ADP on $[^{32}P]$ Ptd OH levels and the consequence of prior addition of adrenaline to platelets pre-treated with flurbiprofen (10 μM). ADP (0.1 - 10 μM; 30s), either alone or when pre-treated with adrenaline (5 μM; 60s) failed to elicit any $[^{32}P]$ Ptd OH formation in human platelets.

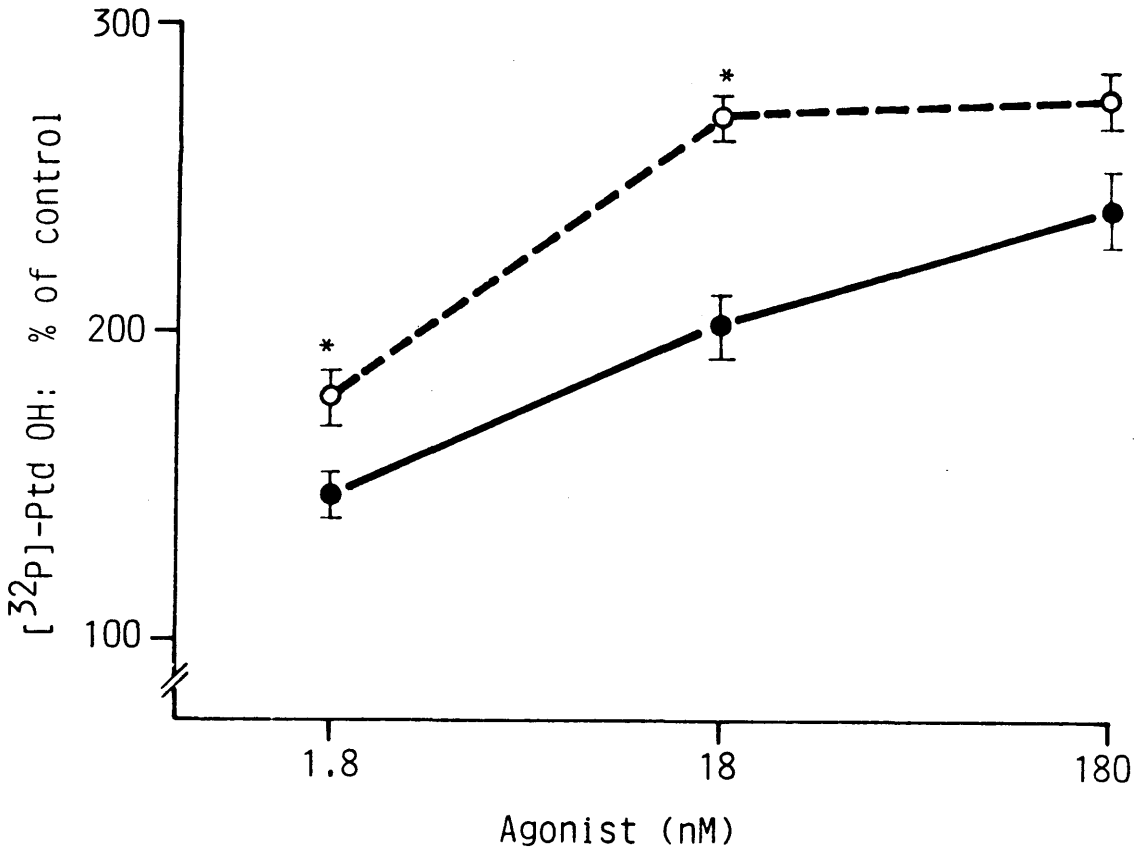

Figure 65. Effect of adrenaline on the concentration response curve for PAF-induced stimulation of $[^{32}P]$ Ptd OH formation.

0.4 ml samples of $[^{32}P]$ P_i prelabelled, flurbiprofen pretreated platelets were pre-incubated for 60s with either saline (closed circles) or 5 μM adrenaline (open circles), prior to the addition of PAF at the concentrations indicated. $[^{32}P]$ Ptd OH levels were measured 30s after PAF addition. The results are means ± S.E.M. of triplicate determinations. The experiment is typical of 3 similar experiments.

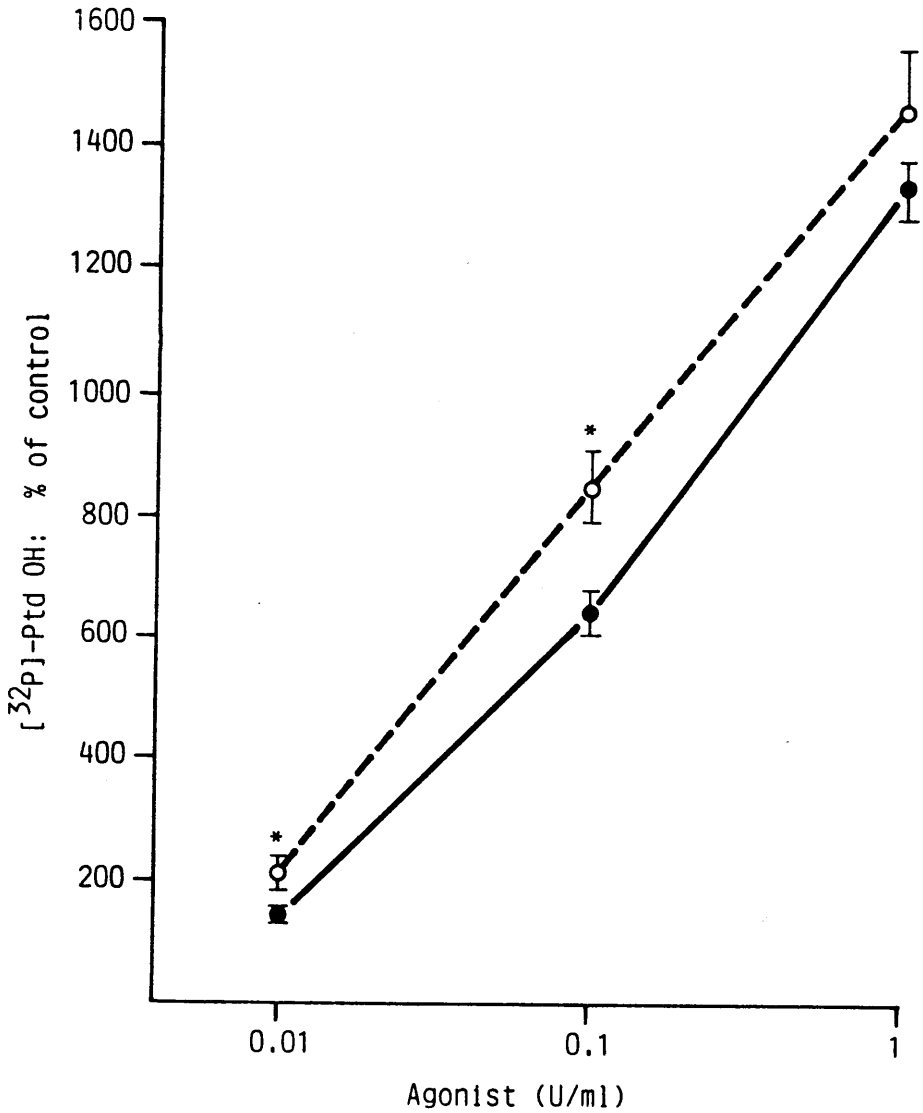

Figure 66. Effect of adrenaline on the concentration response curve for thrombin-induced stimulation of $[^{32}P]$ Ptd OH formation.

0.4 ml samples of $[^{32}P]$ P_i prelabelled, flurbiprofen platelets were pre-incubated for 60s with either saline (closed circles) or 5 μM adrenaline (open circles), prior to the addition of thrombin at the concentrations indicated. $[^{32}P]$ Ptd OH levels were measured 30s after thrombin addition. The results are means ± SEM of triplicate determinations. The experiment is typical of 3 similar experiments. Analysis of data: *$p < 0.05$.

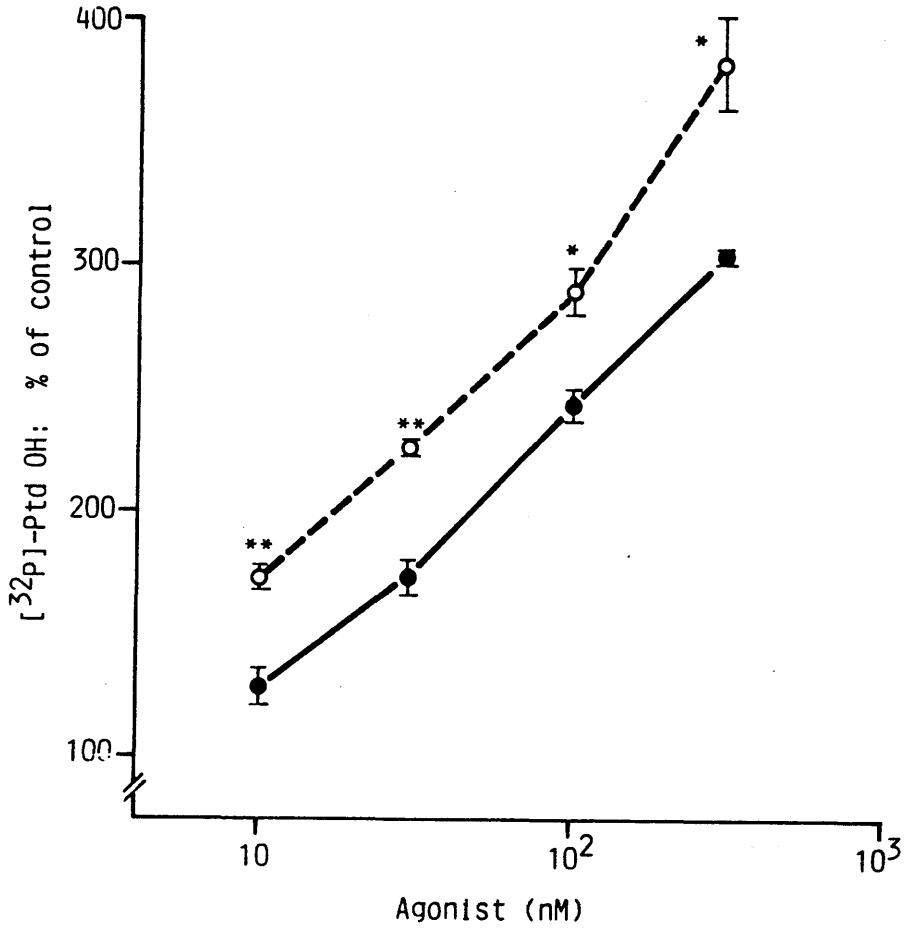

Figure 67. Effect of adrenaline on the concentration response curve for vasopressin-induced stimulation of $[^{32}P]$ Ptd OH formation.

0.4 ml samples of $[^{32}P]P_i$ prelabelled, flurbiprofen pre-treated platelets were preincubated for 60s with either saline (closed circles) or 5 μM adrenaline (open circles), prior to the addition of vasopressin at the concentrations indicated. $[^{32}P]$ Ptd OH levels were measured 30s after vasopressin addition. The results are means ± SEM of triplicate determinations. The experiment is typical of 3 similar experiments. Analysis of data: *$p < 0.05$; **$p < 0.01$.

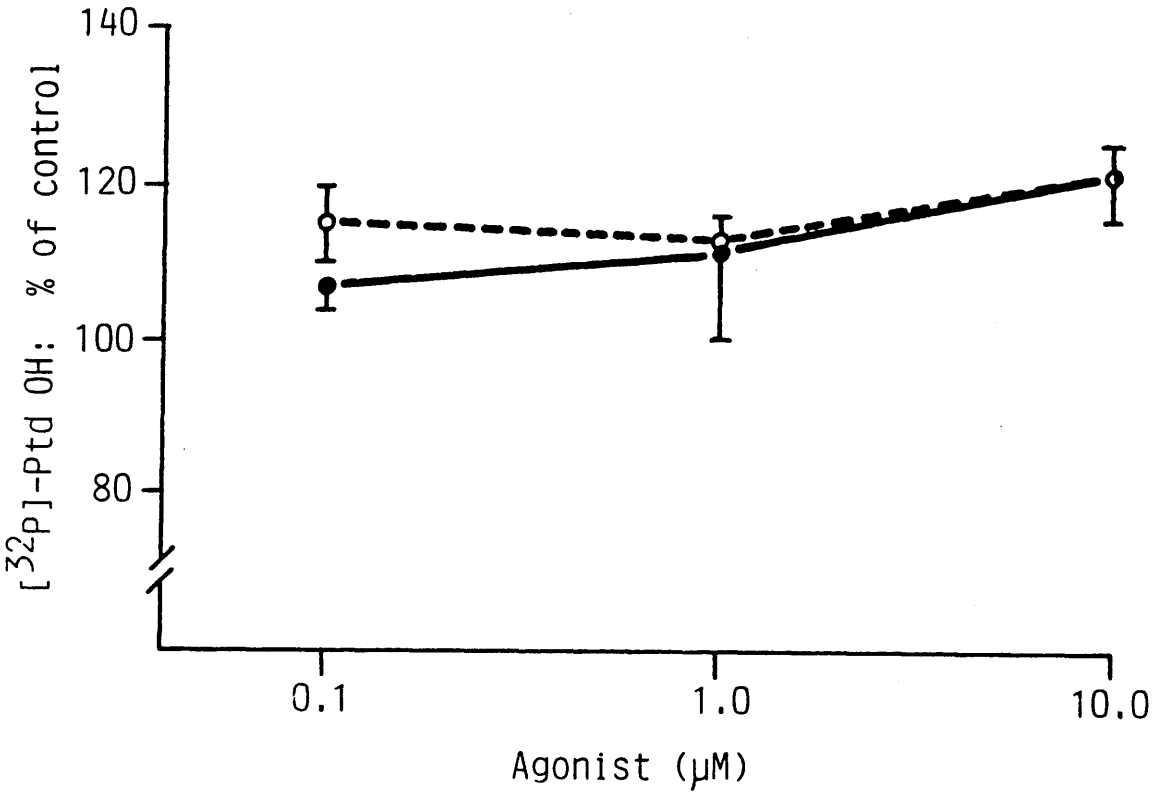

Figure 68. Effect of adrenaline on the concentration response curve for ADP-induced stimulation of $[^{32}P]$ Ptd OH formation.

0.4 ml samples of $[^{32}P]$ P_i prelabelled flurbiprofen pretreated platelets were pre-incubated for 60s with either saline (closed circles) or 5 μM adrenaline (open circles) prior to the addition of ADP at the concentrations indicated. $[^{32}P]$ Ptd OH levels were measured 30s after ADP addition. The results are means ± SEM of triplicate determinations. The experiment is typical of 2 similar experiments.

Adrenaline is known to inhibit adenylate cyclase in intact platelets as it decreases the cellular content of cAMP which has been previously elevated by an adenylate cyclase stimulant. In order to investigate the role of decreased cAMP levels in adrenaline induced potentiation of phosphoinositide hydrolysis, I examined the effect of agents which elevate cAMP on this potentiation.

3.3 Effects of adenylate cyclase stimulants on adrenaline induced potentiation of platelet phosphoinositide metabolism

As adenylate cyclase stimulants inhibit (Figures 42 & 43) and adrenaline potentiates (Figures 65, 66 & 67) agonist induced phosphoinositide hydrolysis it is possible that these agents exert mutually antagonistic effects. This possibility was addressed by examining the effects of adrenaline and adenylate cyclase stimulants (PGD_2, PGI_2) in combination on $[^{32}P]$ Ptd OH formation induced by PAF and vasopressin. Neither of these agonists affect adenylate cyclase activity in intact platelets (Aktories & Jakobs, 1985). PAF (18 nM) elevates the level of $[^{32}P]$ Ptd OH to 295 $\pm$ 15% of the saline control within 30s. This was decreased to 245 $\pm$ 6% of the pre-stimulus level by pre-incubation with 5 µM adrenaline for 60s. PGD_2 added prior to adrenaline blocks the latter's capability to potentiate the PAF induced response to a level (290 $\pm$ 4% of the saline control) which is significantly more than that induced by PAF alone (Figure 69). In a similar fashion, PGI_2 (3 nM; 90s) inhibits adrenaline (5 µM; 60s) induced potentiation of vasopressin (100 nM; 30s) elicited $[^{32}P]$ Ptd OH formation (Figure 70).

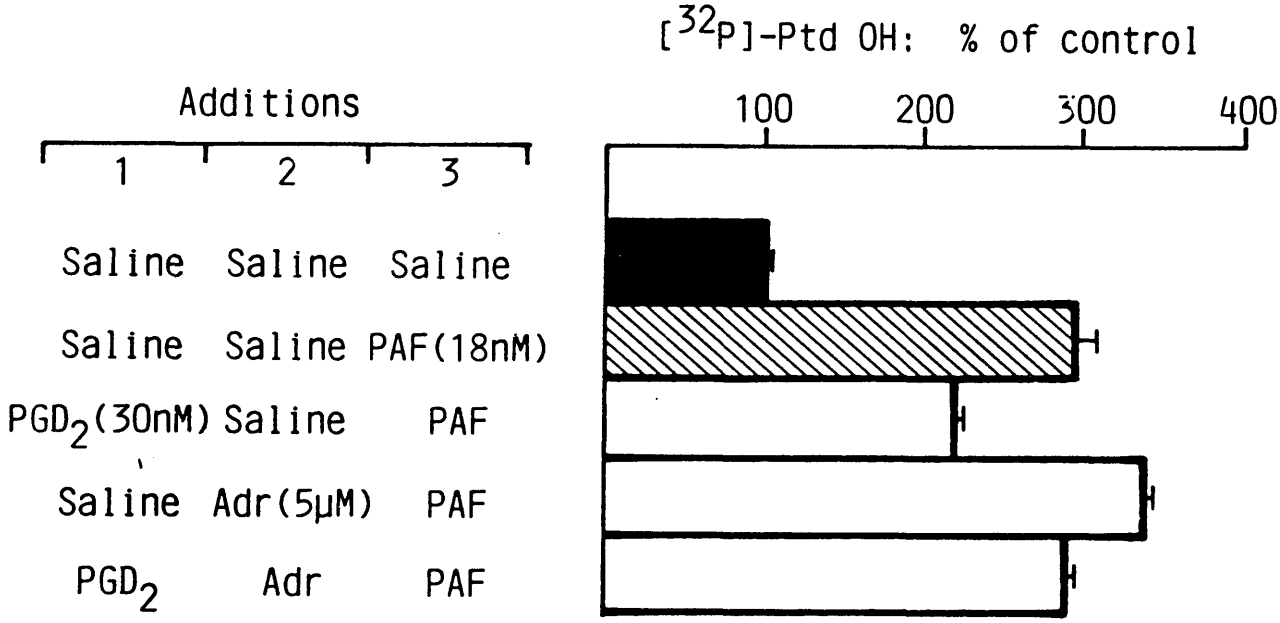

Figure 69. Effect of PGD_2 on adrenaline-induced potentiation of PAF-induced stimulation of $[^{32}P]$ Ptd OH formation.

0.4 ml samples of $[^{32}P]$ P_i prelabelled, flurbiprofen pretreated platelets were sequentially challenged with either saline or 30 nM PGD_2 for 30s, followed by either saline or 5 μM adrenaline for 60s and finally followed by either saline or 18 nM PAF for 30s. The levels of $[^{32}P]$ Ptd OH were measured and the results are means ± SEM of triplicate determinations. The experiment is typical of 2 similar experiments.

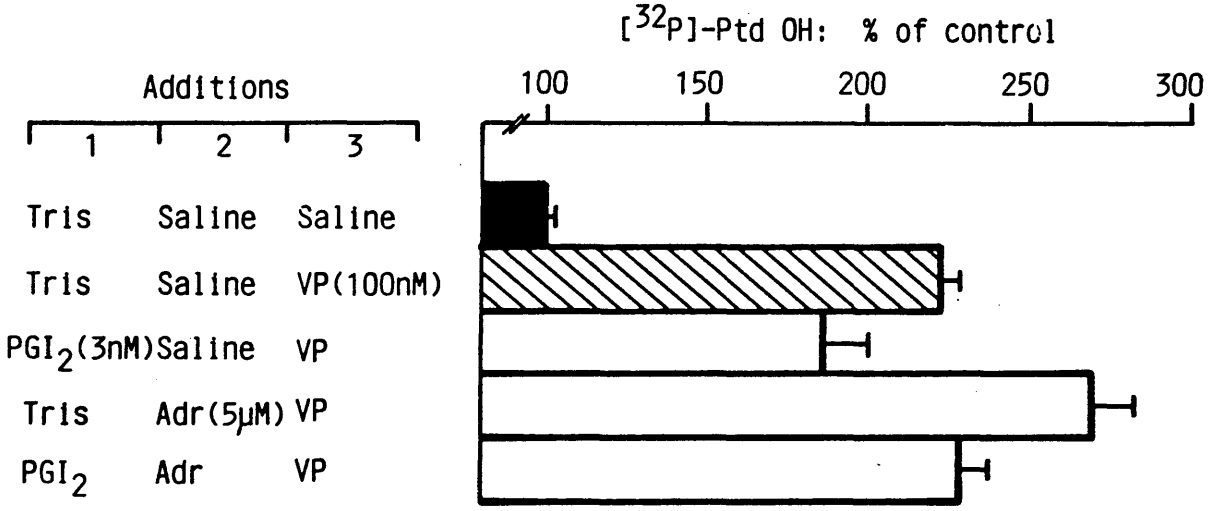

Figure 70. Effect of PGI_2 on adrenaline-induced potentiation of vasopressin-induced stimulation of $[^{32}P]$ Ptd OH formation.

0.4 ml samples of $[^{32}P]$ P_i prelabelled, flurbiprofen pretreated platelets were sequentially challenged with either Tris buffer or 3 nM PGI_2 for 30s, followed by either saline or 5 μM adrenaline for 60s and finally by either saline or vasopressin for 30s. The levels of $[^{32}P]$ Ptd OH were measured and the results are means ± SEM of triplicate determinations. The experiment is typical of 2 similar experiments.

In each case the agonist induced $[^{32}P]$ Ptd OH formation was potentiated by adrenaline pre-treatment and inhibited by adenylate cyclase activation. However pre-incubation with a combination of adrenaline and an adenylate cyclase stimulant had no significant effect on agonist-induced $[^{32}P]$ Ptd OH formation. This suggests that inhibition of adenylate cyclase activity might play a role in adrenaline-induced potentiation of platelet activation by another agonist.

3.4 Summary

Adrenaline and ADP both potentiate agonist induced formation of $[^{32}P]$ Ptd OH. As both agents also potentiate agonist induced platelet functional responses, this effect on platelet phosphoinositide metabolism may underlie the observed effects on functional responses. This potentiation is observed in the presence of flurbiprofen, thus is independent of the formation of PGG_2, PGH_2 or TxA_2. Adrenaline in combination with ADP failed to stimulate formation of $[^{32}P]$ Ptd OH to a detectable level. The adrenaline induced potentiation of agonist induced inositol phospholipid hydrolysis is inhibited by adenylate cyclase stimulants.

DISCUSSION

CHAPTER VIII: DISCUSSION

1. Introduction

The importance of phosphoinositide hydrolysis as a coupling mechanism whereby external stimuli can initiate activation of a variety of cells, including human platelets, has become increasingly apparent in the last decade. The agonist-induced hydrolysis of the inositol phospholipids serves as the/a transduction process by which agonist-receptor interaction at the plasma membrane results in the activation of two distinct second messenger systems; the so-called Ca^{2+} dependent system, utilising $Ins(1,4,5)P_3$ and Ca^{2+}, and the Ca^{2+} independent system, involving DAG. In platelets this stimulation of cellular reactivity elicited by the Ca^{2+}-dependent and Ca^{2+}-independent systems is abrogated by activation of the adenylate cyclase system which leads to the inhibition of cellular reactivity.

This study addressed, not only the role of phosphoinositide hydrolysis in agonist induced platelet stimulation, but also the possibility that putative endogenous regulators could interfere with this hydrolysis. Hence in the following discussion my results will be viewed in the context of current knowledge of the actions and interactions of transduction processes.

2. Mechanism of action of ADP

ADP was the first agonist to be studied with respect to its effects on phosphoinositide hydrolysis in platelets. Several studies, using rabbit platelets, indicated that ADP induced an increased turnover of the inositol phospholipids (Lloyd *et al.*,

1972, 1973a) which was related to shape change rather than to aggregation (Lloyd et al., 1973b). These observations, several years prior to Michell's hypothesis relating this phenomenon to Ca^{2+} gating (Michell, 1975), have been supported in studies monitoring PIP_2 hydrolysis in rabbit platelets challenged with ADP (Vickers et al., 1982; Leung et al., 1983). In addition the same group reported increased phosphoinositide turnover in ADP stimulated human platelets (Vickers et al., 1983). Obviously such observations are at variance with those in this study (Figures 18, 19, 20). However, Fisher and colleagues have reported that, in human platelets, ADP causes an elevation in $[Ca^{2+}]_i$ in the absence of PIP_2 hydrolysis and inositol phosphate formation (Fisher et al., 1985). ADP-induced platelet activation is associated with the phosphorylation of the 20K but not 40K protein (Daniel et al., 1984). These results apparently contradict the hypothesis that agonist induced elevation of $[Ca^{2+}]_i$ occurs as a direct result of the $Ins(1,4,5)P_3$ from phosphoinositide hydrolysis.There are several possible explanations for these discrepancies.

i) It is possible that the techniques employed were insufficiently sensitive to monitor small amounts of ADP induced phosphoinositide hydrolysis in human platelets. This is unlikely however, as agonists which are equally as effective as ADP at stimulating platelets, such as PAF and 5HT (Huang & Detwiler, 1986a), can elicit detectable $[^{32}P]$ Ptd OH formation (MacIntyre et al., 1985b). Moreover the experimental conditions employed in this study were based on those of Lloyd (Lloyd et al., 1972, 1973a,b).

ii) The ADP receptor may become desensitised during the prelabelling period as a consequence of the release of endogenous ADP. However, when desensitisation was prevented by the addition of apyrase during the prelabelling period, ADP remained ineffective at stimulating $[^{32}P]$ Ptd OH formation. Furthermore, under the prelabelling conditions employed, ADP retained the capacity to elicit both an elevation in $[Ca^{2+}]_i$ and functional responses, indicating that the ADP receptor and its transduction processes are intact and that the platelet remains functionally viable.

iii) With a single exception (Vickers et al., 1983), ADP induced phosphoinositide hydrolysis has been observed in non-human platelets. Therefore the discrepancy may be due to a species variation, especially as the reported phosphoinositide hydrolysis in rat platelets (MacIntyre & MacMillan, 1985) utilised identical techniques to those of this study.

iv) Kinetic analysis of the effects of ADP and thrombin in Fura 2 loaded platelets has indicated that these agonists elevate $[Ca^{2+}]_i$ by different mechanisms (Sage & Rink, 1986). In the case of ADP this mechanism may involve the removal of PIP_2 from the plasma membrane and as a consequence the removal of the stimulatory action of PIP_2 on a plasma membrane Ca^{2+}-ATPase (Choquette et al., 1984; Resink et al., 1986). Vickers and colleagues recently demonstrated that, in rabbit platelets, the observed ADP stimulated decrease in PIP_2 levels is not primarily due to phospholipase C activity but to a shift in the equilibrium between PIP_2 and PIP leading to elevated PIP (and Ptd Ins) but decreased PIP_2 levels (Vickers et al., 1986),

presumably by either activation of PIP_2 phosphatase or inhibition of PIP kinase. Although this would serve as a putative mechanism whereby $[Ca^{2+}]_i$ can be elevated in the absence of either Ptd OH or $Ins(1,4,5)P_3$ formation, several anomalies remain. Firstly, neither Fisher et al. (1985) nor this study could monitor decreased levels of PIP_2. It should be noted that the assay technique I used in this study may not be sufficiently sensitive to monitor a small degree of PIP_2 breakdown evoked by "weak" agonists, e.g. PAF, 5HT (Pollock, 1984). Secondly, Vickers et al. (1986) suggested that there is phospholipase C activity directed specifically at Ptd Ins or PIP which results in Ptd OH formation, again contrary to the findings of Fisher et al. (1985) and this study.

v) It is possible that ADP stimulates platelets by an alternative mechanism, independently of the elevation in $[Ca^{2+}]_i$. The ADP receptor has been reported to be linked to N_i, the G protein regulating inhibition of adenylate cyclase. Indeed, in intact platelets ADP reverses the increment in cAMP levels induced by adenylate cyclase stimulants (Mills, 1974). Adrenaline is also believed to be linked to N_i. N_i in turn is linked to inhibition of adenylate cyclase and such an inhibition would result in a decreased intracellular cAMP concentration. Although decreased cAMP levels would, in theory, cause platelet activation (Salzman, 1972), no agonist has been shown to produce such an effect. However distinct compartments of cAMP have been reported in platelets (Hashimoto, 1983) and it is possible that ADP and adrenaline alter the levels of

cAMP within these specific pools, in the absence of changes in the overall cAMP content. It is also possible that the two agonists share an, as yet, unknown mode of action.

Whatever the reason for the observed discrepancies, ADP remains an anomaly amongst stimulatory agonists and further emphasises the complexities and multiplicity of mechanisms underlying platelet activation. Indeed such complexity is not confined to platelets as similar observations have been made in rabbit (Volpi et al., 1984) and human (MacIntyre & Rossi, 1985) neutrophils challenged with leukotriene B_4 where elevated $[Ca^{2+}]_i$ appears unrelated to phosphoinositide hydrolysis.

3. Mechanism of action of thromboxane A_2

Endogenous TxA_2 and its precursors, the prostaglandin endoperoxides PGG_2 and PGH_2, are believed to play a significant pro-aggregatory role in the secondary phase of platelet activation. The natural agonists are highly unstable and/or are converted to other active metabolites. For this reason a large number of stable analogues that mimic the effects of the natural agonists have been utilised to probe their mechanisms of action. The use of one such compound, U44069 (9,11 epoxy-methano PGH_2), demonstrated that the human platelet Tx receptor is linked to inositol phospholipid hydrolysis and elevation of $[Ca^{2+}]_i$ (Pollock et al., 1984). However there is evidence from other tissues that U44069 is a partial agonist at the Tx receptor (Jones et al., 1982).

The novel Tx mimetic, EP 171, stimulated $[^{32}P]$ Ptd OH formation (Figure 21) and ATP secretion (Figure 26) and in both respects was more potent and efficacious than U44069. In addition, EP 171 elicited a much slower stimulation of $[^{32}P]$ Ptd OH formation (Figure 22) and activation of platelets (Jones et al., 1985) than U44069 (Pollock et al., 1984). This slow activation has been attributed to the agonist slowly reaching equilibrium occupation of the receptors as may be expected when a high affinity lipophilic agonist is present at low concentrations. The effects of EP 171 on $[^{32}P]$-PIP_2, -PIP and -Ptd OH levels are further evidence that human platelet Tx receptors are coupled to phospholipase C activity.

The comparable effects of U44069 and the known partial agonist, Et PGH_2 (9,11 ethano PGH_2), in their ability to attenuate the EP 171 induced $[^{32}P]$ Ptd OH formation (Figure 25) and ATP secretion (Figure 27) indicate that, in common with other tissues, U44069 is a partial agonist at the platelet Tx receptor.

This would suggest that EP 171 is a better, i.e. more efficacious or "full", agonist with which to investigate the effects of stimulation of the platelet TxA_2 receptor. However, whether either analogue mimics in all respects the effects of the natural compounds remains to be proven. TxA_2 and PGH_2 have been reported to inhibit platelet adenylate cyclase activity (Miller et al., 1977) whereas U44069 is devoid of this activity (Best et al., 1979). PGH_2 may require to be converted to TxA_2, by Tx synthetase, prior to exhibiting this effect. In contrast, the endoperoxide analogue U44069 is not a substrate for this

enzyme, therefore no TxA_2 would be formed, explaining why U44069 does not inhibit adenylate cyclase (Best et al., 1979). EP 171 does inhibit adenylate cyclase in the platelet, however this is prevented by apyrase, suggesting such an effect is mediated by the release of ADP (MacIntyre & Murdoch, unpublished observations). Whether the effects of endogenous TxA_2 on platelet adenylate cyclase are, likewise, mediated by ADP generation is unclear.

4. Mechanism of action of thrombin

Thrombin is the most efficacious and potent platelet stimulatory agonist and indeed is the archetypical example of a "strong" platelet agonist (Huang & Detwiler, 1986a). It has been shown to instigate the whole range of platelet responses and, as a consequence, has been widely used to probe the stimulatory transduction process present within the cell. In human platelets thrombin has been shown to stimulate:-

i) an elevation of $\left[Ca^{2+}\right]_i$

ii) decreased levels of Ptd Ins, monitored using both prelabelling (Rittenhouse-Simmons, 1979) and mass (Broekman et al., 1980) techniques,

iii) the formation of $\left[^3H\right]$ DAG (Rittenhouse-Simmons, 1979),

iv) increased mass of Ptd OH (Broekman et al., 1980) and, in this study, increased levels of $\left[^{32}P\right]$ Ptd OH (Figures 28, 29),

v) the hydrolysis of PIP_2 (and possibly PIP) (Agranoff et al., 1983; Rendu et al., 1983; Figure 30).

vi) the formation of various inositol phosphates (Agranoff et al., 1983; Watson et al., 1984; Rittenhouse & Sasson, Majerus et al., 1986),

vii) the phosphorylation of a number of proteins, including the 40K and 20K, which have been implicated in platelet activation (Lyons et al., 1975).

These observations strongly suggest a role for receptor mediated phosphoinositide hydrolysis in thrombin stimulated platelet activation. However, the nature of the initial interaction between thrombin and the platelet which triggers this range of responses remains elusive. The functional significance of the thrombin binding site on the platelet plasma membrane has been questioned and a role for proteolysis in thrombin induced platelet activation widely proposed (Martin et al., 1975).

Comparison of the concentration response relationships for the formation of $[^{32}P]$ Ptd OH induced by thrombin (Figure 28) and by the other agonists (Figure 12) clearly demonstrates that thrombin is, by far, the most powerful agonist in this respect. The disparity between the effects of thrombin and other agonists is even more marked when those agonists, which are believed to be functionally significant in activating platelets in vivo, are added in combination and their effects compared to that of thrombin (Figure 31). Thus, it would appear that the thrombin response, which, at the concentrations used in this study, is unaffected by cyclo oxygenase inhibition (Siess et al., 1983a), is not merely the result of the activity of a variety of endogenous mediators (ADP, PAF, 5HT, TxA_2).

The observation that trypsin, apparently in the absence of a specific cell surface receptor, can induce platelet activation (Davey & Luscher, 1967) and formation of $[^{32}P]$ Ptd OH (Figure

32), suggests that platelets can be stimulated by certain proteolytic enzymes. The serine protease inhibitor leupeptin inhibits thrombin and trypsin induced functional responses, protein phosphorylation and $[^{32}P]$ Ptd OH formation (Ruggiero & Lapetina, 1985; Figure 34) but has no effect on $[^{32}P]$ Ptd OH levels elevated by a non-proteolytic agonist, EP 171 (Figure 34). In contrast to receptor specific agonists (e.g. ADP, PAF, 5HT; Figure 31), trypsin can potentiate the formation of $[^{32}P]$ Ptd OH elicited by supramaximal concentrations of TxA_2 (ergo EP 171; Figure 33) and this potentiation is inhibited by leupeptin (Figure 34). This may reflect the situation in thrombin activated platelets, as it demonstrates that it is mechanistically possible to have a twin stimulation via both receptor specific and proteolytic actions. In addition this may help illuminate the vexed question of which inositol phospholipid is hydrolysed during platelet activation. In cells other than platelets there appears to be a consensus that it is PIP_2 which is preferentially hydrolysed by phospholipase C, and Ptd Ins acts solely as a pool replenishing the PIP_2 metabolised (Michell et al., 1981). In contrast, kinetic analysis of thrombin induced phosphoinositide hydrolysis in platelets has indicated that there is direct metabolism of Ptd Ins, as well as of PIP_2 (Wilson et al., 1985c). This has been supported by the observed discrepancy between the mass amounts of DAG and $Ins(1,4,5)P_3$ formed in thrombin stimulated platelets, suggesting that the excess DAG is formed directly from Ptd Ins hydrolysis (Rittenhouse & Sasson, 1985). It is conceivable that this hydrolysis

of Ptd Ins in platelets by thrombin occurs as a direct result of a proteolytic action which is lacking in the effects of agonists in other cell types. It would therefore be instructive to examine whether phosphoinositide hydrolysis induced by weaker platelet agonists, devoid of proteolytic activity such as EP 171 (Figure 33), demonstrate similar kinetic properties and discrepancies in DAG and Ins(1,4,5)P_3 formation to those elicited by thrombin. Such studies may however be impeded by the relative ineffectiveness of these agonists at stimulating phosphoinositide hydrolysis (Figure 12). However monitoring of the effects of trypsin, and other proteases, on the above indices, and the use of specific protease inhibitors on thrombin stimulated platelet activation could circumvent this problem and help elucidate both the mechanism of action of thrombin and the role of direct Ptd Ins hydrolysis in platelet activation. These results imply that caution should be exercised when interpreting studies which use thrombin to probe platelet transduction processes.

5. Desensitisation

It is widely accepted that platelet functional responses exhibit the phenomenon of homologous desensitisation. However the role of inositol phospholipid hydrolysis and elevation of $[Ca^{2+}]_i$ in the underlying mechanism has not been widely investigated.

$[Ca^{2+}]_i$, as monitored by Quin 2 or other indicators, is in dynamic equilibrium and is maintained by a variety of processes; 1) mobilisation from intracellular stores; 2) sequestration to

intracellular stores; 3) influx from the external milieu; 4) extrusion to the extracellular space. Consequently the alteration of any one of these regulatory processes will have profound effects on the $[Ca^{2+}]_i$.

The agonist-induced elevation of $[Ca^{2+}]_i$ is concentration dependent (Figure 37) and believed to reflect both mobilisation and influx of Ca^{2+} (Rink & Hallam, 1984; MacIntyre et al., 1985b). Furthermore the rapidity with which this elevation of $[Ca^{2+}]_i$ declines also is concentration dependent and is perhaps indicative of sequestration and/or extrusion processes being activated to limit platelet activation (Figure 37). In a similar fashion, stimulus-induced formation of $[^{32}P]$ Ptd OH is reversible and the rate of decline increases with agonist concentration (Figure 38). Thus the putative transduction process underlying the elevation of $[Ca^{2+}]_i$ also is negatively regulated by increased agonist concentrations. The mechanism of this regulation may involve either receptor desensitisation or the formation of an endogenous mediator which feeds back to inhibit phosphoinositide hydrolysis and/or activate Ca^{2+} sequestration/extrusion. The latter mechanism may also be implicated in the phenomenon of desensitisation. The addition of agonists to platelets, prechallenged with the same agonist, fails to elicit any further formation of Ptd OH (Figure 39). This has been widely reported for platelet aggregation (e.g. O'Brien, 1966; Evans & Gordon, 1974; Ruggles & Scrutton, 1979) and recently for elevation of $[Ca^{2+}]_i$ (Pollock & MacIntyre, 1986), and has been attributed to receptor mediated, homologous desensitisation. However, if the second agonist differs from

the first, then the evoked [^{32}P] Ptd OH formation was less than that elicited by the second agonist if added alone (Figure 39) perhaps indicative of heterologous desensitisation. These effects are comparable to those observed in blowfly salivary glands (Berridge & Fain, 1979) which was presumed to result from a depletion of the inositol phospholipids, as it was reversible by the subsequent addition of free inositol to allow de novo synthesis of the lipids (Fain & Berridge, 1979). However, such a mechanism can be precluded in the present study as thrombin can elicit much more Ptd OH formation than combination of other agonists (Figures 31, 39). This indicates that there is more phospholipid precursor present than is utilised by other agonists, but this does not exclude the possibility of either, specific pools of phosphoinositides which are sensitive to individual agonist-induced phospholipase C activity, or, the "clumping" of receptors thereby limiting their accessibility to the membrane lipids (Berridge & Fain, 1979), as mechanisms of heterologous desensitisation. It is also possible that the levels of G protein in the membrane is a limiting factor. This however is unlikely as in other cells there is an excess of G proteins present (Rodbell, 1980). Another possibility is the production of endogenous mediators which function to inhibit the platelet stimulatory transduction process(es). Obvious candidates for such agents are the platelet inhibitory mediators, cAMP and cGMP.

5.1 Adenylate cyclase stimulants

Adenylate cyclase stimulants, such as PGI_2 and PGD_2, inhibit agonist induced platelet functional responses (Haslam et al., 1980; Bushfield et al., 1985), elevation of $[Ca^{2+}]_i$ (Feinstein et al., 1983; Bushfield et al., 1985; Figure 40), phosphoinositide hydrolysis (Rittenhouse-Simmons, 1979, Figures 42, 43) and protein phosphorylation (Lyons et al., 1975). Kaser-Glanzmann and colleagues proposed that the inhibitory effects of cAMP were due to its stimulation of the uptake of Ca^{2+} into various intracellular membranes (Kaser-Glanzmann et al., 1977). Such an action of cAMP is consistent with the ability of stimulants of adenylate cyclase accelerating the rate of decline of an agonist induced elevation of $[Ca^{2+}]_i$ (Zavoico & Feinstein, 1984; Figure 56). However, such a mechanism has been challenged as cAMP was found to be ineffective at sequestering Ca^{2+} into the DTS (Menashi et al., 1982) and its inhibitory action was proposed to be independent of its effects on $[Ca^{2+}]_i$ (Pannocchia & Hardisty, 1985). PGI_2 has been shown to prevent agonist induced formation of $Ins(1,4,5)P_3$ (Watson et al., 1984), indicative of an inhibitory effect at, or prior to, the level of phospholipase C, although the precise site of cAMP mediated inhibition is unclear. The platelet functional responses are differentially sensitive to increments in platelet cAMP levels. The most sensitive responses (ATP secretion and aggregation) most probably are inhibited by an effect at a level distal to second messenger generation, whereas inhibition of shape change occurs only at levels of cAMP that also inhibit elevation of Ca^{2+} and Ptd OH formation (Bushfield et al., 1985). A cAMP-

dependent protein kinase mediated alteration of receptor structure (phosphorylation) and inhibition of G protein function and consequent impairment of phospholipase C activity are all other possible mechanisms.

PGI_2 and PGD_2 can reverse agonist induced elevation of $[Ca^{2+}]_i$ (Figure 56) and formation of $[^{32}P]$ Ptd OH (Figure 58) respectively, in a manner similar to increasing agonist concentration. This may indicate that cAMP can act to limit or terminate agonist induced platelet activation. Consequently cAMP may serve as a putative endogenous mediator of desensitisation. However, the effects of PGD_2 on elevated $[^{32}P]$ Ptd OH levels cannot be reproduced by the use of the stable cAMP analogue, 8 Br cAMP (Figure 60). Corresponding studies on an agonist induced elevation in $[Ca^{2+}]_i$ cannot be carried out in intact cells as during the period required for incorporation of the stable analogue (five minutes) the levels of Ca^{2+} would have returned to basal levels. At the concentrations used in this study, PGD_2 is not entirely specific for adenylate cyclase (MacIntyre et al., 1985a), and so its effects on elevated $[Ca^{2+}]_i$ and/or $[^{32}P]$ Ptd OH may not be solely mediated by cAMP. In addition platelet stimulatory agonists do not increase the cellular levels of cAMP (Haslam et al., 1980) as may be expected were it to play a regulatory role in platelet function.

5.2 Guanylate cyclase stimulants

Cyclic GMP is believed to be an intracellular messenger for various extracellular signals in a number of cells although

the presence of substrates for cGMP dependent protein kinase(s) have only rarely been detected. The cGMP-dependent protein kinase shows similar catalytic properties to those of the cAMP-dependent protein kinase and so cGMP may provide a negative rather than positive stimulus (Nishizuka, 1984). Agents which stimulate guanylate cyclase (NaNP) or mimic cGMP (8 Br cGMP) cause muscle relaxation and so cGMP has been implicated as a feedback inhibitor, rather than a positive messenger, of muscarinic stimulated smooth muscle contraction (Schultz et al., 1977), thereby providing an immediate control preventing the cell from over-responding. In general, in cells, such as platelets, where cAMP inhibits the effects of stimulatory agonists, cAMP and cGMP are not mutually antagonistic, but rather similarly inhibit receptor mediated transduction processes and functional responses (Nishizuka, 1984). NaNP has a powerful inhibitory effect on human platelets (Glusa et al., 1974). Moreover it has been reported that platelets synthesise cGMP in response to stimulatory agonists such as collagen, thrombin, adrenaline, ADP and 5HT (White et al., 1973; Haslam & McClenaghan, 1974; Davies et al., 1976). This possibility occurs as a consequence of arachidonate liberation (Davies et al., 1976) although whether cyclo-oxygenase activity is a pre-requisite for cGMP formation is unclear (Davies et al., 1976; Best et al., 1979). On these grounds it has been proposed that cGMP is an endogenous negative mediator of platelet activity (Haslam et al., 1980) and thus may be involved in the mechanisms underlying desensitisation.

NaNP and/or 8 Br cGMP inhibit agonist induced elevation in $[Ca^{2+}]_i$ (Figure 40; Nakashima et al., 1986), phosphoinositide hydrolysis (Figure 44; Takai et al., 1981b; Nakashima et al., 1986) and protein phosphorylation (Takai et al., 1981b). This indicates that cGMP inhibits platelet activation at, or before, the level of phospholipase C. In addition, cGMP can terminate the agonist induced stimulation of the transduction process, as indicated by the reversal of agonist induced elevation of $[Ca^{2+}]_i$ (Figure 56) and formation of $[^{32}P]$ Ptd OH (Figure 59). In contrast to cAMP stimulants, it appears that, at the concentrations used, NaNP is fairly specific for guanylate cyclase (MacIntyre et al., 1985a) and, in the case of Ptd OH reversal, mimicked by the stable analogue, 8 Br cGMP (Figure 60). This effect of cGMP to inhibit the formation of, and stimulate the removal of, stimulatory second messengers is further evidence for and consistent with a negative feedback role for this nucleotide within the cell. However, whether such a mechanism is physiologically relevant or is involved in the process of desensitisation in platelets is open to conjecture.

5.3 Protein kinase C stimulants

It has become evident that agonist-receptor interaction initiates a cascade of events, including the generation of two distinct second messengers, Ca^{2+} and DAG, which culminate in the cellular response (Berridge & Irvine, 1984). DAG, which is believed to activate a cytosolic enzyme, protein kinase C (Nishizuka, 1983) and cytosolic free Ca^{2+} may act independently or synergistically to initiate a variety of cellular responses

in a host of cells (Nishizuka, 1984).

The tumour promoting class of compound, the phorbol esters, such as PMA, have been shown to mimic the stimulatory effects of DAG on protein kinase C (Castagna et al., 1982). However in contrast to the transient nature of DAG-mediated protein kinase C stimulation, phorbol esters permanently activate the enzyme, although recently it has been reported that DAG may be elevated for a longer period than originally believed (Duncan & Lloyd, 1986) perhaps resulting in a PMA-like activation of protein kinase C. Agents such as 4 β phorbol and 4 α phorbol didecanoate are structurally similar to the active phorbol esters and share many of their physico-chemical properties, but differ in that they do not activate protein kinase C (Castagna et al., 1982) and are widely used as controls to distinguish protein kinase C-mediated from non-specific effects of phorbol esters in experimental situations.

The phorbol esters are potent activators of numerous cells including platelets (Zucker et al., 1974; White et al., 1974). They have the capacity to stimulate ATP secretion in the absence of elevated $[Ca^{2+}]_i$ (Rink et al., 1983) and also demonstrate a marked synergism with calcium ionophore in promoting degranulation (5HT release) (Yamanishi et al., 1983), arachidonic acid release (Halenda et al., 1985) and Tx biosynthesis (Mobley & Tai, 1985). The release of arachidonic acid is believed to occur as a result of a protein kinase C-mediated phosphorylation of a protein (lipocortin) with anti phospholipase A_2 activity (Touqui et al., 1986). This phosphorylation removes the inhibitory constraint

with the consequent liberation of arachidonic acid. Phorbol esters also stimulate neutrophils (De Chatelet et al., 1976). However, in contrast to the predicted synergism between protein kinase C activation and elevation of $[Ca^{2+}]_i$, they were shown to activate an ATP-dependent calcium extrusion mechanism (Mottola & Romeo, 1982) and to attenuate an agonist induced elevation in $[Ca^{2+}]_i$ (Lagast et al., 1984).

In this study phorbol esters were shown to have a similar pronounced inhibitory action on an agonist induced increase in $[Ca^{2+}]_i$ in human platelets. Although the active compound, PMA, has no effect on the resting $[Ca^{2+}]_i$, it both inhibits (Figure 45) and reverses (Figure 56) an agonist induced elevation in $[Ca^{2+}]_i$. This regulatory effect has subsequently been confirmed (Zavoico et al., 1985; Poll & Westwick, 1986) and found to be directly dependent on the preincubation time (Krishnamurthi et al., 1986). Suppression of the agonist-induced elevation of $[Ca^{2+}]_i$ could perhaps be attributed to an activation of an extrusion process analogous to that thought to exist in the neutrophil. However as no reduction in the resting cytosolic calcium levels was observed then, either no such mechanism exists in the platelet, or it is only activated in the presence of elevated $[Ca^{2+}]_i$. The 40K protein has recently been shown to act as an $Ins(1,4,5)P_3$ phosphatase (Connolly & Majerus, 1986) which would result in the removal of this inositol phosphate. Consequently, this would decrease the ability of the cell to mobilise calcium. Moreover PMA stimulates the dephosphorylation of $Ins(1,4,5)P_3$ (Molina y Verdia & Lapetina,

1986), an effect consistent with protein kinase C stimulating the removal Ins(1,4,5)P_3 and this would partly explain the inhibitory effects of PMA on an agonist-induced elevation of $[Ca^{2+}]_i$.

Phorbol esters also appear to interfere with phosphoinositide hydrolysis. PMA stimulates both the $[^{32}P]$ labelling (Figure 36; de Chaffoy de Courcelles et al., 1984a; Halenda & Feinstein, 1984) and the mass amounts (Halenda & Feinstein, 1984) of PIP and PIP_2, although PIP significantly more so than PIP_2. These alterations in the levels of the poly phosphoinositides were accompanied by a decrease in the $[^{32}P]$ content (Figure 35; Halenda & Feinstein, 1984) and mass (Halenda & Feinstein, 1984) of Ptd Ins. In contrast, there is no alteration in the resting cellular level of Ptd OH (Figure 35; Halenda & Feinstein, 1984), indicating that phosphodiesteric cleavage of inositol phospholipids was not stimulated. However, the phorbol esters inhibit the agonist induced formation of $[^{32}P]$ Ptd OH (Figures 47-54). Were PMA to inhibit phospholipase C, then this would explain both the suppression of $[^{32}P]$ Ptd OH formation and attenuation of Ca^{2+} flux. However as monitoring of Ptd OH formation is but an indirect index of phospholipase C activity, such an extrapolation is unwarranted. Inhibition of $[^{32}P]$ Ptd OH formation is also consistent with the inhibition of DAG kinase. However, PMA inhibits the agonist induced hydrolysis of PIP_2 (Figure 55; Zavoico et al., 1985; Watson & Lapetina, 1985) and formation of inositol phosphates (Watson & Lapetina, 1985; Rittenhouse & Sasson, 1985). This evidence indicates that

inhibition at or before the level of phospholipase C, with the consequent suppression of PIP_2 hydrolysis and impaired $Ins(1,4,5)P_3$ formation, can account at least in part for the observations made in this study.

Protein kinase C allegedly mediates the so-called "calcium independent" mechanism of cellular activation. Therefore the observation that a stimulant of the enzyme acts to limit or terminate the production of stimulatory second messengers appears to be somewhat incongruous. Although the phosphorylation of the 40K protein, an indicator of protein kinase C activity, was not measured, in the present study, the experiments were performed using concentrations of PMA and preincubation times consistent with complete activation of the enzyme (Castagna et al., 1982; Yamanishi et al., 1983; Kaibuchi et al., 1983). In addition, the synthetic analogue of DAG, 1-oleoyl-2-acetyl-glycerol (OAG), has been shown to activate protein kinase C in a manner analagous to natural diglycerides and to be metabolised to the corresponding Ptd OH (Kaibuchi et al., 1983). These agents have been shown to increase the platelet levels of PIP and PIP_2 (de Chaffoy de Courcelles et al., 1984b), to inhibit the formation of inositol phosphates (Watson & Lapetina, 1985) and dephosphorylate $Ins(1,4,5)P_3$ (Molina y Vedia & Lapetina, 1986) in a manner similar to PMA. Moreover, H7 (1- 5-isoquinolinesulphonyl -2-methylpiperazine), the proposed protein kinase C inhibitor (Kawamoto & Hidaka, 1984), has been shown to prevent the inhibition of an agonist induced elevation of $[Ca^{2+}]_i$ by phorbol esters (Tohmatsu et al., 1986; Poll &

Westwick, 1986) and the hydrolysis of inositol phospholipids (Tohmatsu et al., 1986). Whilst none of these observations provide conclusive proof that inhibitory effects of phorbol esters are mediated by protein kinase C, they do provide some evidence that the observed effects are not a result of a non-specific action on the cell.

Interestingly, in human platelets the phorbol esters have an inhibitory effect on the adenylate cyclase system. PMA inhibits PGD_2 but not PGI_2 mediated accumulation of cAMP (Bushfield et al., 1986) indicating both that their effects have a measure of selectivity and the complex nature of the interactions between transduction processes in the platelet.

If the effects of phorbol esters mimic those of endogenous DAG and are due to an action on protein kinase C, then this enzyme may play a bi-directional regulatory role in various cells. Indeed such a dual role for DAG has been proposed to be an elegant mechanism whereby the $[Ca^{2+}]_i$ can be regulated within certain limits (Drummond, 1985). In human platelets, phorbol esters and/or DAG have been reported to inhibit agonist induced ATP (Watson & Lapetina, 1985), arachidonic acid and β-thromboglobulin release (Krishnamurthi et al., 1986). Inhibition of 5HT secretion (Watson & Lapetina, 1985) which is antagonised by H7 (Poll & Westwick, 1986) has also been reported, however this has been challenged (Krishnamurthi et al., 1986). Consequently the functional significance in terms of platelet reactivity remains contentious.

The effects of the phorbol esters on the cellular levels of phosphoinositides, discussed above, have been similarly reported in other cells. In lymphocytes, PMA enhances the $[^{32}P]$ incorporation into PIP and PIP_2 with the corresponding decreased incorporation into Ptd Ins (Boon et al., 1985). The effects of phorbol esters on agonist elevated $[Ca^{2+}]_i$ has subsequently been demonstrated in a host of isolated cells including rat basophilic leukaemia cells (Sagi-Eisenberg et al., 1985), rabbit neutrophils (Naccache et al., 1985), hepatocytes (Cooper et al., 1985), astrocytoma cells (Orellana et al., 1985), GH_3 pituitary cells (Drummond, 1985), vascular smooth muscle (Brock et al., 1986) and adrenal glomerulosa cells (Kojima et al., 1986). Similarly phorbol esters have inhibitory effects on agonist induced phosphoinositide hydrolysis in cells other than platelets. Rat hippocampal slices (Labarca et al., 1984), astrocytoma cells (Orellana et al., 1985), mast cells (Okano et al., 1985), lymphocytes (Mellors et al., 1985), vascular smooth muscle (Brock et al., 1985; McMillan et al., 1986), hepatocytes (Corvera et al., 1986) and adrenal glomerulosa cells (Kojima et al., 1986) all exhibit this phenomenon. The variety of cells in which there is evidence for an inhibitory action of phorbol esters on their agonist stimulated inositol phospholipid metabolis and consequent elevation of $[Ca^{2+}]_i$ suggests that this is a frequent occurrence and thus may be of physiological significance.

Phorbol esters have also been found to influence other receptor-linked transduction processes, in particular the

adenylate cyclase system. For example PMA inhibits the stimulation of adenylate cyclase activity in rat hepatocytes by glucagon (Heyworth et al., 1984; Garcia-Sainz et al., 1985). Such an observation would suggest that the effects of phorbol esters were not specific for phospholipase C.

As discussed above there is evidence for protein kinase C stimulants (phorbol esters and OAG) acting to inhibit agonist-induced phosphoinositide metabolism at, or prior to, phospholipase C. Decreased levels of the enzyme substrates (the phosphoinositides) could account for the observed effects, however as PMA promotes both the $[^{32}P]$ levels and mass of PIP and PIP_2 this is unlikely. The activity of the 40K protein as an $Ins(1,4,5)P_3$ phosphatase most likely accounts at least in part for the observed effects on $[Ca^{2+}]_i$.

However phorbol esters apparently affect the adenylate cyclase, as well as the phospholipase C, mediated system, and therefore it is possible that they act at a level which is present in both processes, such as receptors and/or G proteins. The phorbol esters have been shown to affect agonist receptor interaction. $Alpha_1$ adrenoceptors are phosphorylated in smooth muscle (Leeb-Lundberg et al., 1985) and their affinity for agonists is reduced in hepatocytes (Corvera et al., 1986) and, in addition, muscarinic acetylcholine receptors on neuroblastoma cells are rapidly internalised (Liles et al., 1986) by phorbol esters. The insulin receptor has been reported to be phosphorylated by phorbol esters (Jacobs et al., 1983) and PMA and OAG both induce desensitisation to β-adrenergic agonist stimulation

in the adenylate cyclase system of rat reticulocytes, an effect, mediated at the receptor level and which is antagonised by H7 (Yamashita et al., 1986). There are as yet no reports of phorbol esters influencing the number or structure of receptors in the human platelet. The phorbol esters may act on some common site or subunit of the various G proteins present in the membrane. They have been shown to have an action at the level of the phospholipase C linked G protein consistent with preventing thrombin induced activity (Halenda et al., 1986). However the precise site of the negative regulation of cellular reactivity and various transduction processes by protein kinase C activation (by DAG and/or PMA) is still unclear.

Therefore there is some evidence that activation of protein kinase C may play a functional role as a bidirectional regulator of cellular activity. As well as stimulating the cell, protein kinase C may feed back inhibit the stimulatory transduction process and so avoids overstimulation of the cell. The DAG which is produced in response to agonists may have a role to play in the desensitisation of the human platelet.

6. Potentiation

Human platelets in vivo are exposed to a wide number of endogenous compounds (e.g. collagen, ADP, PAF, adrenaline, TxA_2) which are believed to play a role in initiating or propagating the coagulatory process. These agonists exert a synergistic effect on platelet reactivity. However the precise mechanism(s) underlying this effect have not been widely investigated.

The majority of studies have concentrated on the potentiation by ADP or adrenaline on thrombin, 5HT and PAF induced platelet functional responses. The former two agonists have been shown to be linked via N_i to the inhibition of adenylate cyclase in intact human platelets, whereas thrombin, 5HT and PAF are believed to be mediated by phosphoinositide hydrolysis. As such this represents a means of evaluating the contribution of distinct transduction processes to the resultant cellular response.

Adrenaline, which itself neither elevates $[Ca^{2+}]_i$ nor causes inositol phospholipid metabolism (MacIntyre et al., 1985b), significantly potentiates the effects of PAF, vasopressin and thrombin on human platelet phosphoinositide hydrolysis, at least as monitored as $[^{32}P]$ Ptd OH formation (Figures 65-67). Concomitant with this, adrenaline similarly enhances the increase in $[Ca^{2+}]_i$ elicited by an agonist (Bushfield et al., 1987). This effect of adrenaline on agonist induced $[^{32}P]$ Ptd OH levels was antagonised by the prior addition of the adenylate cyclase stimulants PGD_2 or PGI_2 (Figures 69, 70).

The mechanism of action of ADP is the subject of much conjecture. ADP can elevate $[Ca^{2+}]_i$ (MacIntyre et al., 1985b) but is apparently ineffective at stimulating phosphoinositide metabolism (Figures 18-20). In common with adrenaline, ADP can potentiate PAF and vasopressin induced $[^{32}P]$ Ptd OH formation (Figures 63, 64).

Studies using calcium ionophores and phorbol esters have indicated that neither increased elevation of $[Ca^{2+}]_i$ nor increased DAG (and so Ptd OH) levels alone could account for

the observed potentiation by adrenaline of agonist induced platelet activity. However, a combination of both, as would be expected if phospholipase C activity were enhanced, could account for the observed effects (Bushfield et al., 1987). Such an action could occur were adrenaline or ADP, either as a result of occupancy of their specific receptor or due to a reduction in a specific intracellular cAMP pool to promote the binding of phospholipase C mediated agonist to their receptors. This could be achieved were adrenaline and ADP to alter the arrangement of the membrane lipids (Irvine, 1982). Alternatively, an enhanced coupling, or efficacy of the coupling process, of G proteins could account for the observed potentiation. The structure of the putative phospholipase C linked G protein is unclear. This G protein in platelets has been reported to be different from either of those in the adenylate cyclase system (Houslay et al., 1986). Although thrombin has been reported to be linked to two G proteins the first is apparently N_i and the second apparently novel (Grandt et al., 1986). However, in rabbit neutrophils (Volpi et al., 1985) and human leukemic cells (Brandt et al., 1985), activation of phospholipase C is inhibited by pertussis toxin. As this toxin also inhibits N_i to which adrenaline and ADP receptors are linked in platelets, it is possible that the phospholipase C linked G protein is similar to N_i. It is possible that as a consequence of this similarity, when ADP or adrenaline bind to their receptors, N_i enhances the coupling of the phospholipase C linked G protein to the enzyme thereby promoting its activity.

7. Concluding remarks

This study was concerned primarily with the role of phosphoinositide hydrolysis in the stimulation of the human platelet, and its role in the development of desensitisation and the putative mechanism of potentiation within the cell. The study has failed to elucidate the mechanism of action of ADP in stimulating human platelets and the enigma of how an elevation in $[Ca^{2+}]_i$ can occur in the apparent absence of phosphoinositide hydrolysis remains. It appears that there are receptor operated calcium channels present in the cellular membrane which are linked to the ADP receptor but which are independent of phosphoinositide hydrolysis. It appears that this is not unique to platelets as it also occurs in LTB_4-induced neutrophil activation, and further studies in the platelet are required to identify the nature of these channels. In common with others, this study demonstrates that thrombin induced platelet activation is not solely as a result of agonist receptor interaction and indicates the need for caution when using this agonist to investigate transduction process within the cell. The investigations involved with putative mechanisms underlying desensitisation and potentiation have demonstrated the intricate and complicated interactions of the adenylate and guanylate cyclase and the inositol phospholipid systems within the cell. Further studies in platelets, and other cells, are required to clarify these interactions and to elucidate any functional role they may play.

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