"CYTOSOLIC FREE CALCIUM AND PLATELET RESPONSES TO PUTATIVE LIPID MEDIATORS OF PLATELET ACTIVATION"

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BY

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Adenosine diphosphate (ADP) Sigma.
Arachidonic acid Sigma.
Aspirin Boots.
Bovine Serum Albumin Sigma.
8-Bromo-cyclic adenosine monophosphate (8-Br cAMP) Sigma.
8-Bromo-cyclic guanosine monophosphate (8-Br cGMP) Sigma.
BW775C Wellcome research laboratories.
Dazoxiben (UK 37-248-01) Pfizer.
8-(N,N-diethyl-amino)octyl-3,4,5-trimethoxybenzoate (TMB-8) - was a gift from Dr. E. W. Salzman Harvard Medical School.
Digitonin Sigma.
Diltiazem Sigma.
Ethylene diaminotetra-acetic acid (EDTA) Sigma
Ethylene glycol-bis(B-amino-ethyl ether) N',N'-tetraacetic acid (EGTA) Sigma.
Flurbiprofen (froben) Boots
Imipramine Ciba-Geigy.
3-Isobutyl-1-methyl-xanthine (IBMX) Sigma.
Methoxyverapamil Knoll
Nicardipine Yamanouchi Pharmaceutical Co.
Palmetoyl-lysophosphatidic acid (LPA) P & L Biochemicals Inc.
Platelet activating factor (PAF)
- was a gift from Dr. saunders, Sandoz. Inc.
Prostaglandin I_2 Sigma.
Prostaglandin D_2 Sigma.
Quinacrine Sigma
Quin2-acetoxymethyl ester Quin2-AM Lancaster Synthesid Ltd.
Sepharose 2B Sigma.
Serotonin Sigma.
Sodium nitroprusside Sigma.
TMB-8
Thrombin Sigma.
Thromboxine B_2 Upjohn.
Trimethoquinol (TMQ) Roche.
U44069 Upjohn.
Vasopressin Sigma.
Verapamil Knoll.

Radiochemicals

^{3}H -adenosine 3',5' cyclic monophosphate. Amersham.
^{14}C -5 hydroxytryptamine. Amersham.
^{3}H-platelet activating factor. New England Nuclear.
^{125}I -succinyl cyclic AMP tyrosine methyl-ester. Amersham.
^{125}I -succinyl cyclic GMP tyrosine methyl-ester. Amersham.
^{3}H -thromboxane B_2 New England Nuclear.

"Primary" platelet aggregation is usually reversible and is mediated solely by an exogenous agonist. "Secondary" platelet aggregation is irreversible and is initiated by an exogenous mediator but propagated by endogenous mediators synthesised (eg. TXA_2, PGG_2, PGH_2) and/or secreted (eg. ADP, 5HT) by activated platelets. Activated platelets also synthesis two phospholipids 1-O-alkyl-2-acetyl-glycerol-3-phosphorylcholine (PAF; PAF acether) and lysophosphatidic acid (LPA) which are potent inducers of platelet activation and hence may also serve as endogenous mediators of human platelet activation. These various agonists interact with specific recognition sites or receptors in/on the platelet. Agonist-receptor interaction influences the intracellular concentrations of biochemical intermediaries. These so-called "second messenger" molecules (eg. Ca++, cAMP, 1,2-diacylglycerol) influence the rates of those very biochemical reactions that govern the cellular response. At the commencement of this study no systematic examination of the effects and/or mechanism(s) of action on platelets of putative lipids mediators of platelet activation (PAF, LPA and TXA_2) had been conducted. Consequently my initial studies were concerned with assessing:-

1. The effects of PAF, LPA, TXA_2 (using U44069, a TXA_2 mimetic) on platelet aggregation, degranulation and TXB_2 biosynthesis.

2. The role of endogenous arachidonate metabolites and ADP in the above responses.

Thereafter, using the fluorescent Ca++ indicator dye quin2, I

examined the role, the sources and the pharmacology of elevated cytosolic free calcium concentration ($[Ca^{++}]_i$) in agonist-induced platelet activation.

In platelet rich plasma PAF (10 - 100nM), U44069 (10 - 100nM) and LPA (1 - 30 μM) induced "primary" aggregation whereas higher concentrations elicited "secondary" aggregation, degranulation and (except for U44069) TXB_2 biosynthesis.

Agents (aspirin, dazoxiben, trimethoquinol and 13-azaprostanoic acid) that inhibit the formation or action of PGG_2, PGH_2 and TXA_2 impaired PAF- and LPA-induced aggregation and degranulation. In this respect the thromboxane synthetase inhibitor dazoxiben was much less effective than inhibitors of cyclooxygenase or endoperoxide/TXA_2 receptor antagonists. It was concluded that PAF and LPA-induced secondary aggregation and degranulation depend upon an intact cyclooxygenase but not thromboxane synthetase. The ADP receptor antagonist (beta-gamma methylene ATP) suppressed "secondary" aggregation but not degranulation or TXB_2 formation elicited by PAF or LPA. Consequently, endogenous ADP is important in mediating phospholipid-induced secondary aggregation.

Resting $[Ca^{++}]_i$ in quin2 loaded platelets was estimated to be 90 $\pm$ 3 nM The platelet stimulatory agonists PAF, LPA, U44069, thrombin, ADP, 5HT and vasopressin but not adrenaline induced a concentration-dependent elevation of $[Ca^{++}]_i$ to around 500nM. Chelation of extracellular Ca++ using EGTA markedly attenuated the elevation of $[Ca^{++}]_i$ induced by all agonists, indicating that the major source of elevated $[Ca^{++}]_i$ derives via influx of

external Ca++. Moreover the effects of PAF and ADP on 5HT release were markedly attenuated by removal of external Ca++ whereas the effects of thrombin were only slightly reduced. This suggests that for PAF and ADP elevated $[Ca^{++}]_i$ is an important mediator/determinant of 5HT release, but is less important for the effect of thrombin.

Depolarization of the platelet trans-membrane potential using high $[K^+]_o$ = 112nM failed to elevate $[Ca^{++}]_i$ or alter the extent of the elevation of $[Ca^{++}]_i$ induced by agonists. In addition, the dihydropyridine Ca++ channel agonist BAY K8644 failed to elevate $[Ca^{++}]_i$ or alter responses to agonists. It is concluded that Ca++ influx across the plasma membrane occurs via channels that open as a consequence of agonist-receptor interaction - receptor operated channels (ROCs) rather than as a consequence of changes in membrane potential - voltage operated channels (VOCs).

Calcium channel blockers (CCBs) are agents that prevent the influx of extracellular Ca++ and recently, based on $[^3H]$-nimodipine binding studies, have been separated into three distinct classes . To determine whether the ROC associated with particular platelet agonists was sensitive to a particular class of CCB the inhibitory effects of nicardipine (class I), verapamil (class II) and diltiazem (class III) were examined against submaximal primary aggregation induced by PAF, LPA, U44069, ADP and vasopressin. Aggregation induced by all agonists was inhibited in a concentration-dependent manner by the CCBs. However, with the exception of diltiazem which

potently inhibited the response induced by PAF and U44069 (I_{50} = 9 uM and 15 μM respectively), and verapamil which potently inhibited the response induced by PAF (I_{50} = 22 ± 4 μM), inhibition was only observed with high concentrations of CCBs (> 100μM). Hence it would appear that the ROC associated with the PAF receptor is sensitive to class II and class III, whereas the ROC associated with the U44069 receptor is only sensitive to class III CCB.

When the inhibitory effect of verapamil and diltiazem was monitored against submaximal elevations of $[Ca^{++}]_i$ induced by PAF and U44069 an identical inhibitory profile was observed. However both diltiazem and verapamil abolished the elevation of $[Ca^{++}]_i$ completely. Hence verapamil and diltiazem are capable of inhibiting both Ca++ influx and Ca++ mobilization. Indeed, when monitored under conditions of $[Ca^{++}]_o$ = 1mM and $[Ca^{++}]_o$ = 0mM (ie. in the presence of 3mM EGTA), the concentration response curve for inhibition, by verapamil, of the elevation of $[Ca^{++}]_i$ induced by PAF were identical.

The nature of the selective inhibition of PAF-induced platelet responses by verapamil was further examined. Verapamil inhibited , apparently competitively, PAF-induced platelet aggregation, elevation of $[Ca^{++}]_i$ and phosphoinositide hydrolysis - believed to be the molecular mechanism underlying agonist-induced elevation in $[Ca^{++}]_i$. These effects on aggregation, and $[Ca^{++}]_i$ were shared by the quaternary derivative of verapamil (D575) which is normally inactive as a CCB unless injected into the cell. It is concluded that the effects of verapamil are due to an action at the outer surface

of the platelet plasma membrane and could be attributed to a competitive interaction at the putative PAF receptor.

It is known that agents which stimulate adenylate or guanylate cyclase inhibit platelet functional response to all agonists. Several studies have shown that cAMP can enhance Ca++ uptake by platelet membrane fractions and in smooth muscle the inhibitory response of cGMP is thought to be due to redistribution of Ca++ i. Hence the inhibitory effect of PGI_2, PGD_2 (adenylate cyclase stimulants), Na nitroprusside (a guanylate cyclase stimulant) and 8-bromo-cAMP and 8-Bromo-cGMP was compared on platelet aggregation, and on elevations of $[Ca^{++}]$ i induced by PAF. The rank order of potency for inhibition of aggregation was identical to that for inhibition of $[Ca^{++}]$ i (ie. PGI_2 > PGD_2 > NaNP >8Br cGMP > 8Br cAMP) suggesting that supression of $[Ca^{++}]$ i availability may be the mechanism underlying the action of cAMP and cGMP.

Agonist-induced elevations of $[Ca^{++}]$ i in platelets is transient, suggesting that there exists homeostatic mechanisms for Ca++ sequestration and/or extrusion. Addition of PGI_2, PGD_2, or NaNP at the peak of the elevation of $[Ca^{++}]$ i induced by PAF resulted in an enhanced rate of Ca++ restoration. It has been reported that cGMP may be produced by platelets following exposure to stimulatory agonists and this could act as an endogenous regulatory of elevated $[Ca^{++}]$ i. However no elevation of platelet cGMP or cAMP was observed for up to 5 min. following the addition of PAF, thrombin or U44069 to platelets. It is concluded that cGMP may serve as a negative feedback regulator of elevated $[Ca^{++}]$ i in platelets, and that other

endogenous molecules eg. 1,2-diacylglycerol may also subserve such a role.

It is clear that changes in $[Ca^{++}]_i$ alone are unlikely to be the sole determinant of platelet reactivity. Nevertheless a knowledge, firstly of the processes that govern $[Ca^{++}]_i$ in platelets and secondly, of how these may be interfered with pharmacologically, should facilitate the choice or design of therapeutic agents to limit platelet reactivity.

1. PLATELET STRUCTURE, FORMATION AND LIFE CYCLE.

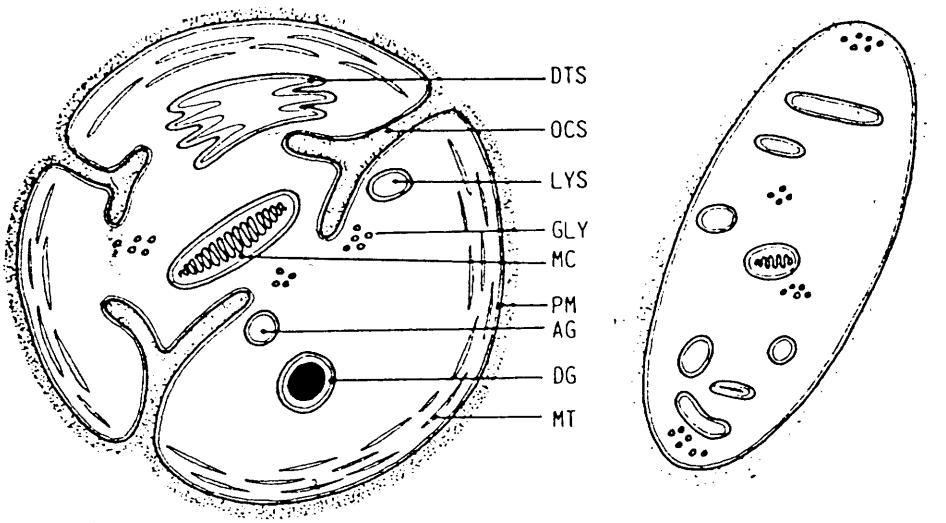


Figure 1a. Diagramatic representation of platelet ultrastructure viewed in equatorial and longitudinal cross-section. MT - microtubules; MC - mitochondria; PM - plasma membrane; DG - dense granules; AG - alpha granule; LYS - lysosomes; OCS - open canalicular system; GLY - glycogen granules.

1.1. MORPHOLOGY

The resting platelet is discoid in shape, 2-3μm in diameter and 0.7μm in thickness and is the smallest of the formed elements of the blood.

The main structural features of this cell are illustrated in figure 1a. The plasma membrane is composed of a lipid bilayer with protein inclusions , similar in structure to other blood cells and rich in glycoproteins. A band of microtubules forms a peripheral ring around the equatorial plane and is thought to serve as a cytoskeleton responsible for maintaining the shape of the quiescent cell. **Phillips et. al. (1983)** have also described the existence of an extensive microfilament system which may also contribute to the platelet architecture.

The dense tubular system is derived from endoplasmic reticulum and consists of internal membranes subjacent to the plasma membrane. This system may function in calcium metabolism **(Statland et. al., 1969).**

The open canalicular system is a series of invaginations of the plasma membrane and is responsible for the sponge like appearance of the platelet in electron micrographs. These invaginations increase the surface area of the platelet and maintain a close connection between the internal and external environment. The function of this system is unclear but may facilitate exocytosis.

Platelets contain three populations of storage granules: electron dense granules (dense bodies), alpha-granules and lysosomes. In addition platelets also contain glycogen granules and a few mitochondria.

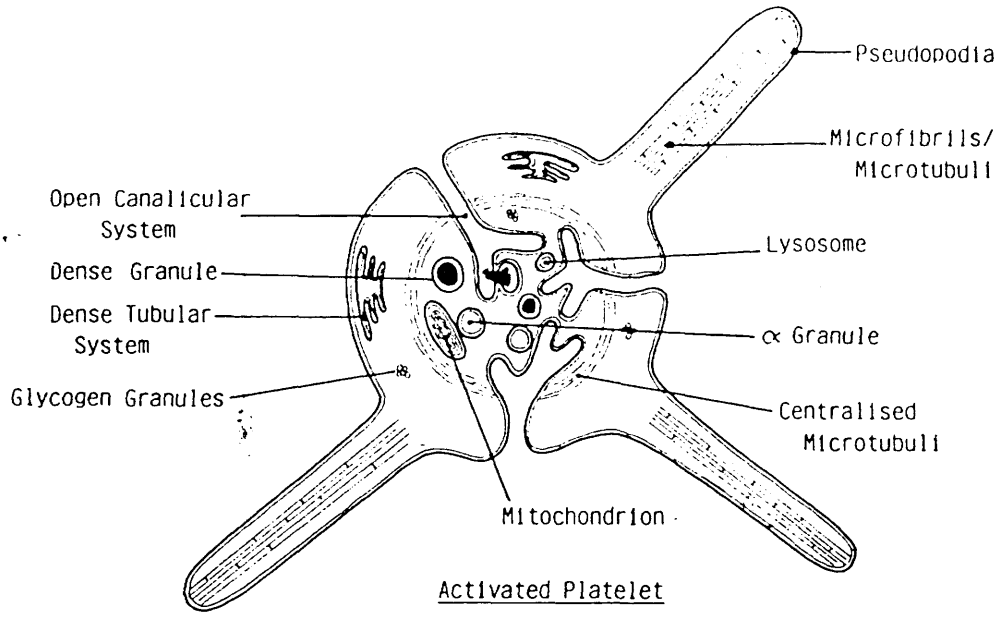


Figure 1b. Diagramatic representation of activated platelet

Once activated platelets rapidly lose their disc shape and become spherical with protruding pseudopodia (figure 1b.). Such gross alterations in the platelet profile are thought to be due to structural changes in the underlying cytoskeleton. In platelets the cytoskeleton has two functions, structural and contractile. The structure of the quiescent platelet is apparently maintained by the microtubules and also perhaps the microfilament system. Platelet activation is associated with clustering of internal granules in the centre of the platelet surrounded by a ring of microtubules and microfilaments. In addition both filamentous systems are present in the pseudopodia. Clearly a reorganization of the cytoskeleton occurs upon platelet activation. The actual sequence of events has not yet been established, however centralization of the cytoskeleton is though to be due to contraction of the existing cytoskeleton and responsible for granule clustering. Depolymerization of the cytoskeleton is also thought to occur followed by formation of a restructured cytoskeleton, this event may be responsible for the formation of pseudopodia. (see section 2.2.2a.

1.2. ORIGIN AND LIFE CYCLE

The blood platelet is an anucleate cell with only a vestigil protein synthesizing ability. Consequently most of the structual and functional components must be derived from the progenitor cell, the megakaryocyte.

Megakaryocytes were first recognised as the platelet progenitor by **Wright (1906)** based purely on morphological similarities between the two cell types. Since then much evidence has accumulated supporting a platelet megacaryocyte "axis". For example thrombocytopenia is accompanied by an increase in the size and number of megakaryocytes **(Harker & Finch, 1969)**, conversely thrombocytosis suppresses megakaryocytopoiesis. Megakaryocytes and platelets share common antigens **(Humphrey, 1955; Vazques & Lewis, 1960; de Leval, 1967)**, display similarities in chemical composition **(Jackson, 1973; Darzynkiewiez etal, 1967; Rabellino et al, 1979)** and isotopic labelling of the megakaryocyte leads to subsequent labelling of the circulating platelets **(Odell & McDonald, 1964; Pennington, 1969; Najean & Ardaillou, 1969).**

Platelets are formed by the division of the cytoplasm of megakaryocytes, large polyploid cells (20-50μm) found predominantly in the bone marrow but also in lung, liver and heart **(Smith & Butcher, 1952).** Formation of individual platelets in the megakaryocyte occurs in an orderly process. Synthesis of platelet components, assembly and accumulation are reflected in the different stages of megakaryocyte maturation. Three distinct steps are recognized. The megakaryoblast is

marked by intense endomitosis accompanied by internalization and proliferation of the plasma membrane which ultimatly divides the megakaryocyte cytoplasm into individual platelets, the so-called demarcation membranes. This process begins in the earliest identifiable megakaryocyte **(MacPherson, 1971; Levine, 1980)**. Also characteristic of this stage is intense protein synthesis **(Behnke, 1970)**. The golgi apparatus is highly active and thought to be involved in mucopolysaccharide synthesis **(Tavassoli, 1979)** an essential component of the plasma membrane. Granule formation is less evident at this stage. As the megakaryocyte matures increasing RNA synthesis is characteristic of the second stage. This is observed histologically by increased basic staining. In the basophil megakaryocyte cell organelles are now prominent, granule formation is also evident and concentrated in the vicinity of the golgi apparatus **(MacPerson, 1972; Geyer & Schaaf, 1972)**. These granules exhibit histological similarities with alpha-granules of circulating platelets **(Breton-Gorius & Guichard, 1975)**. Since alpha-granules appear to be formed in the megakaryocyte it is also likely that the granule proteins, such as B-thromboglobulin and platelet derived growth factor, are synthesised by the megakaryocyte. Indeed some platelet specific proteins have been identified in the megakaryocyte **(Castro-Malaspina et. al., 1981)**. Smooth endoplasmic reticulum is present and is histochemically identical to the dense tubular system of the platelet **(Breton- Gorius & Reyes, 1976)**. Also, so-called platelet specific glycoproteins have been isolated from megakaryocytes **(Rabellino et al, 1981)**. Towards the end of this stage basophilic staining diminishes

and endomitosis ceases.

The megakaryocyte is the fully differentiated member of the series, although microtubules and dense tubule fragments are still randomly distributed and do not exhibit the orderly structures associated with platelets, probably indicating that platelets are not yet fully formed. The precise process whereby platelets are liberated by the mature megakaryocyte is unclear. Evidence suggests that megakaryocytes form pseudopodia which penetrate the blood sinusoids releasing segments of cytoplasm into the blood **(Becker & de Bruyn, 1976; Tavassoli, 1979).** These segments or proplatelets are elongated structures of about 2.5 x 120μm and it has been estimated that the mature megakaryocyte may produce up to six proplatelets each proplatelet giving rise to as many as one thousand platelets **(Becker, 1976).** The process of fragmentation probably takes place in the circulation. The precise location is unknown although the microcirculation of the lung has been suggested as a likely area **(Trowbridge et. al., 1983).** Apparently it is also possible for the entire megakaryocyte to pass the marrow-blood barrier and enter the circulation **(Kinosita & Ohno, 1958).** This seems to be the source of the pulmonary megakaryocytes where, because of their size, they are trapped in the microcirculation. Again this may be the location of fragmentation **(Trowbridge et. al., 1983).**

The control of megakaryocyte maturation is still poorly understood, but a humoral substance(s) in the blood termed thrombopoietin has the ability to stimulate immature megakaryocytes, increasing ploidy and size **(Odell et. al., 1976).** The level of thrombopoietin varies inversely with the

circulating platelet concentration **(Odell & Murphy, 1974).** Therefore in thrombocytopenia the level of this hormone probably increases and when the normal platelet count is attained the thrombopoietin level probably decreases leading to a parallel fall in the rate of megakaryocyte maturation and hence platelet production.

The circulating platelet concentration is normally around 2×10^8/ml. A number of techniques have been employed to assess the life-span of the circulating platelet. Of these $[^{51}Cr]$-labelling appears to be the most reliable and indicates a period of about 10 days **(Harker & Finch, 1969).** Senescent platelets are removed from the circulation by the reticuloendothelial system. The mechanism by which this system recognises that platelets are ready for removal is unclear although **Greenberg et. al. (1975)** have found that lowering the platelet sialic acid content results in immediate clearence from the circulation. Incorporation into a haemostatic plug is also responsible for removing platelets from the circulation and consequently the activity of the haemostatic process would obviously influence the platelet life-span.

2. BLOOD PLATELETS: CELLULAR REACTIONS AND BIOLOGICAL FUNCTION.

2.1. PLATELET REACTIONS.

Considering their size and anucleate nature it hardly seems surprising that blood platelets, for so long, were viewed merely as preparation artifacts. Yet for the same reasons it seems remarkable that these cells are capable of such a wide variety of cellular reactions. Such reactivity makes the platelet unique among the blood cells.

2.1.1. ACTIVE TRANSPORT

In their quiescent state platelets actively transport 5-hydroxytryptamine (5HT), adenine, adenosine and taurine from the plasma **(Gordon & Milner, 1967)**. Indeed virtually all the plasma 5HT is sequestered in the dense bodies of these cells. 5HT uptake is energy dependent and occurs against a considerable concentration gradient **(Lingjaerde, 1969; Sneddon, 1969; Shaskan & Snyder, 1970)**. Two separate uptake mechanisms are involved in this process: one, located in the plasma membrane, is responsible for transport from the plasma to the cytosol; the second, located in the granule membrane, transports the amine from the cytosol into the dense bodies **(Pletscher et. al., 1978; Rudnick et. al., 1980)**. Adenine and adenosine are accumulated by different specific uptake processes **(Sixma et. al., 1976)** and incorporated into the cytoplasmic nucleotide pool. Platelets have two nucleotide compartments **(Ugurbil & Holmsen, 1981)** that are virtually non-exchangable. The cytoplasmic pool which provides the energy

requirements for the cell, and a pool sequestered in the dense bodies. The latter is secreted during the release reaction. The function of taurine uptake is at present unknown.

2.1.2. PHAGOCYTOSIS

Like white cells circulating platelets may be capable of phagocytosis and could therefore contribute to the removal of particulate material from the blood **(Aken & Vreeken, 1969)**.

2.2. RESPONSES TO STIMULI.

The unique role of platelets DEPENDS in the morphological and biochemical changes that occur during stimulation. In response to physiological stimuli platelets develop the following properties and hence participate in the associated cellular reactions.

1. They adhere to foreign surfaces (adhesion).

2. They lose their discoid shape and become spherical with protruding pseudopodia (shape change).

3. They unmask membrane phospholipids (exposure of pro-coagulants).

4. They cohere with other platelets (aggregation).

5. They secrete biologically active constituents from storage granules (release reaction).

6. They generate and release active metabolites of arachidonic acid (icosanoid biosynthesis).

7. Display various manifestations of active contraction (clot retraction).

Platelets reactivity can be stimulated by a number of physiological agonists and can be inhibited by agents such as PGI_2, PGE_1, & PGD_2. Specific membrane receptors are responsible for recognising the external stimulus and for initiating the processes that translate the extracellular stimulus into the appropriate cellular response. Furthermore the cohesive properties of the activated cell stems from the

exposure of specific receptors for fibrinogen or lectins on the plasma membrane. The principle component of these receptors are membrane glycoproteins. Thirty or more glycoproteins are associated with the plasma membrane of platelets and the role of some of these glycoproteins, in receptor expression, is discussed in sections 2.2.6.

The above reactions bestow upon the platelet considerable biological versatility and collectively dictate the principle function of the blood platelet. Platelets are involved in inflammation, wound healing and certain pathological conditions, however haemostasis is the one physiological process that depends exclusively on the platelet. In the event of vascular injury platelets adhere to the subendothelium. Platelet aggregates form on the carpet of adherent cells resulting in the formation of a primary haemostatic plug. Activated platelets accelerate several reactions in the coagulation pathway thus promoting fibrin deposition in the primary plug. Finally the loose meshwork of platelets and fibrin condenses to form a clot culminating in the cessation of bleeding. Activation of this system in the absence of any apparent physiological motive is responsible for the pathological condition of thrombosis.

The role of platelets in inflammation has not been fully assessed. An increase in vascular permeability and the accumulation of leukocytes, at the site of tissue damage, is characteristic of the inflammatory response. Platelets release various factors which have the potential to mediate both phenomena. Moreover since platelets accumulate at the site of

tissue damage they may play a significant role in mediating this process. Platelet mitogens may also contribute to the genesis of atherosclerosis.

2.2.1.ADHESION.

One of the unique reactions the of platelet is their ability to adhere to a variety of "foreign" or more correctly thrombogenic surfaces. In vivo the endothelial lining of the vasculature constitutes a nonthrombogenic surface. Consequently platelets circulate as quiescent cells. In the event of endothelial discontinuity, for example from vascular trauma, platelets adhere to the subendothelial connective tissue **(Hugues, 1960; Zucker & Borelli, 1962).** Connective tissue consists of microfibrils of elastin, basement membrane, and collagen fibrils. Of these, platelets have the highest affinity for fibrillar collagen **(Mayer & Weisman, 1978; Cazenave et. al., 1973).** However it is not absolutely clear whether or not adhesion involves a classical receptor-ligand interaction. Observations that collagen, extracted from activated platelets, was associated with fibronectin **(Bensusan et. al., 1978)** prompted the hypothesis that this glycoprotein may be the collagen receptor on platelets. Fibronectin is found in the plasma and on the surface of cells including platelets and is also secreted by platelets **(Bensusan et. al., 1978; Plow et. al., 1978).** Subsequent studies using anti-fibronectin antibodies have shown that inhibition of this glycoprotein has little effect on adhesion **(Santoro & Cunningham, 1979).**
Adhesion also requires a plasma cofactor Von Willebrand factor (VWf) (part of the Factor VIII complex). Platelets from blood lacking this factor (patients with Von Willebrand's disease) display poor adhesion **(Tschopp et. al., 1974). Kao et.**

al. (1979) have shown that quiescent platelets have a low affinity for VWf. In contrast VWf binding to the subendothelium correlates with platelet adhesion **(Sakariassen et. al., 1979).** It has been proposed therefore, that a subendothelial component binds VWf causing a subsequent allosteric alteration enabling this protein to recognise a specific receptor on the platelet **(Kirby, 1977; Kao et. al., 1979).**

Ristocetin is an antibiotic which induces platelet agglutination only in the presence of VWf **(Howard & Firkin, 1971).** A number of studies indicate that the ristocetin-dependent VWf receptor on platelets is glycoprotein 1b on the plasma membrane **(Berndt & Phillips, 1981).** Ristocetin could therefore mimic the endothelial component by causing the same allosteric alteration in VWf allowing it to bind to platelets.

Platelet adhesion is the first stage in the formation of a haemostatic plug. Collagen not only acts as a binding matrix but is also a potent secretagogue (see platelet secretion 2.2.4).

2.2.2. PLATELET SHAPE CHANGE.

Shape change is one of the early manifestations of the platelet response. In cell suspensions this reaction is characterised by a rapid change from discs, in the quiescent state, to spheres with protrusions of pseudopodia in the activated state. The function of shape change is not completely clear although pseudopodia are thought to facilitate aggregation and are probably involved in clot retraction. A shape change also occurs in adherent cells where they spread over the thrombogenic surface, however this is a much slower response than that observed in the cell suspension.

While such changes involve gross alterations in the membrane contour, the underlying mechanism appears to be a reorganization of the fibrillar cytoskeleton with the plasma membrane responding passively.

Platelets contain two of the three fibrillar systems of eukaryotic cells: microtubules and microfilaments. These systems have two apparent roles in platelets - structural and contractile.

2.2.2a. ULTRA-STRUCTURAL EVENTS ASSOCIATED WITH PLATELET ACTIVATION: ROLE OF MICROTUBULES AND MICROFILAMENTS

Microtubules are present as an equatorial band in the resting platelet **(White, 1971)** and appear to function in maintaining the discoid shape of the cell. The link between microtubules and cell shape was first suggested by **Fawcett (1959)** in an article on bullfrog erythrocytes. Subsequent observations that certain mammalian erythrocytes (Camel & Llama) which have an ovoid structure contained a marginal bundle of microtubuli while other mamalian erythrocytes, devoid of structure, had no such system support this view **(Barclay, 1966).**

In platelets direct evidence for a structural role for this organelle became apparent using techniques which selectively destroy microtubules. Microtubules may be classified into two major groups : "stable" & "labile" **(Wilson & Bryan, 1974).** Stable microtubules, typified by those seen in cilia and flagella, are temperature insensitive - that is they do not depolymerize on cooling. In contrast labile microtubules, seen in the spinacle lattice of dividing cells and in the dendrites of nervous tissue, are extremely temperature sensitive and unlike stable microtubules are also sensitive to anti-mitotic agents such as colchicine. Platelet microtubules fall into the latter group. Consequently the function of this organelle may be simply resolved by its selective destruction either by cooling or treatment with an anti-mitotic. Under such conditions quiescent platelets lose their disc shape and become

irregular or spherical **(Behnke, 1970).**

What happens to microtubules upon platelet stimulation?

Electron micrographs have revealed a great deal of information on this topic. Unlike cooled platelets, where the origional coil can reform on warming **(Behnke, 1970)**, physiological stimulation results in a reorganisation of the microtubules. The initial event appears to be contraction of the equatorial band, since following stimulation a centralized band is observed surrounding the intracellular organelles. Later parallel bundles of microtubules are observed in the pseudopodia indicating a depolymerization and reorganisation of the original structure. Biochemical evidence, measuring colchicine binding also supports the conclusion that microtubules break down and later reform **(Steiner & Ikeda, 1979).**

The available evidence supports a structural role for microtubules in the resting platelet. Although bundles of microtubules are observed in the pseudopodia they are unlikely to be involved in pseudopod formation since pseudopod formation is unaffected by colchicine treatment **(Boyle-Kay & Fudenberg, 1973).**

Microfilaments or actin containing filaments are present in the platelet in two forms - polymerized as filaments (F-actin) or in the monomeric form (G-actin). The cytoplasm within the microtubule ring is dense and granular and therefore prohibits the observation of filamentous material. Consequently it is difficult to establish whether this system contributes to the shape of the resting cell. **Nachmias (1983)** believes that in

the resting cell actin exists predominantly in the monomeric form which polymerizes on activation. **Phillips et. al. (1983)** have also shown that polymerization occurs on stimulation. In contrast however, the latter group believe that an extensive fibrillar network, consisting of 40 - 50 % F-actin, exists in the resting cell which increases to 60 - 80 % upon activation. Indeed these workers have extracted, from resting cells, such a network which retains retains a discoid shape. Although fibrillar material cannot be observed in the resting cell electron micrographs of activated cells show the microfilaments, like microtubules, as a bond surrounding the centralized organelles and extensivly in the pseudopodia.
The work of **Phillips et. al. (1983)** presents a possible structural role for the microfilament system in the resting cell. Microfilaments are present in the pseudopodia, again no firm evidence links microfilaments to pseudopod formation, however, some of the studies by Nachmias tentatively indicates a positive role in pseudopod formation.

Shortly after the initial shape change there is a reorganisation of the cytoplasmic contents. Platelet granules are centralised within the cytoplasm. Because the centralised granules are surrounded by a band of microtubules and microfilaments it has been proposed that microtubules and/or microfilaments are responsibe for this event. It has been suggested that such a movement brings the granules into intimate contact with the open canalicular system into which they are thought to secrete their contents **(Sixma, 1974).**

The kinetics of this movement preceeds or co-incides with degranulation **(Skaer, 1981).**

Microtubules play an important role in insulin secretion from the pancreatic B-cells and in the secretory activity of the thyroid, parathyroid, anterior pituitary, polymorphonuclear leukocytes and macrophages **(Dustin, 1978).** Although it has not been established whether granule centralization is causally related to exocytosis or whether this event is indeed mediated by microfilaments or microtubules it is thought that activation of the contractile elements of these systems sweeps the cytoplasmic organelles in a centripetal motion.

2.2.3. UNMASKING MEMBRANE PHOSPHOLIPIDS.

The involvement of membrane phospholipids in the coagulation process has been reviewed recently **(Zwaal, 1978).** Platelet activation is associated with profound changes in membrane lipids. Among these changes a phospholipid procoagulant complex, termed platelet factor 3 (PF3), is unmasked on the outer plasma membrane. PF3 accelerates the formation of thrombin from prothrombin and the convertion of factor X to Xa. These reactions require the formation of an activated intermediate complex comprising the various enzyme/substrates. PF3 is thought to provide a matrix which allows factors X, V and prothrombin to bind with the correct conformation forming the so-called prothrombinase complex, and similarly factors IX and VII to form the factor X activating complex.

PF3 is thought to be a negatively charged phospholipid. Platelet phospholipids may be classified according to the charge on their polar head groups. At neutral pH phosphatidylcholine (PC) phosphatidylethanolamine (PE) and sphingomylin (SPM) have no net charge while phosphatidylserine (PS) and phosphatidylinositol (PI) are negatively charged. In the resting platelet the negatively charged PS and PI are almost exclusively located in the inner leaflet of the plasma membrane whereas the outer leaflet is composed of neutral phospholipids **(Zwaal & Bevers, 1983).**

Platelet stimulation is associated with a transbilayer movement of PS. Consequently the outer membrane surface becomes

negatively charged. The mechanism(s) underlying this movement is unclear but probably occurs as a consequence of shape change. PF3 activity has also been extracted from the alpha-granule membrane. Hence part of the PF3 activity may be derived from fusion of the alpha-granule with the plasma membrane.

In exposing PF3 the platelet can:-

a) promote and confine coagulation to the imediate vicinity of the injury.

b) consolidate the haemostatic plug through fibrin deposition.

c) propagate the haemostatic response by promoting thrombin formation.

Dense bodies:-	ATP (adenosine triphosphate)	
	ADP (adenosine diphosphate)	
	pyrophosphate	
	ionic calcium	
	5HT (5'hydroxytryptamine)	
Alpha granules:-	fibrinogen	
	factor V	
	thrombospondin	
	fibronectin	
	platelet factor 4	(PF4)
	low affinity platelet factor 4	(LAPF4)
	B-thromboglobulin	(BTG)
	platelet derived growth factor	(PDGF)
	platelet basic protein	(PBP)
	vascular permeability factor	
	albumin	
Lysosomes:-	B-N-acetyl glucosaminidase	
	B-galactosidase	
	B-glucuronidase	
	B-glycerophosphatase	
	elastase	

Table 1. Constituents secreted from platelet dense bodies, alpha granules and lysosomes.

2.2.4. PLATELET SECRETION.

Platelets display two types of secretion. They can selectively release the contents of intracellular storage granules (release reaction) and generate and release metabolites of arachidonic acid. Secretion is an important event in the haemostatic process but also accounts for much of the biological versatility of these cells, allowing them to participate in such events as inflammation, wound healing and atherogenesis.

Release reaction : the constituents released from platelet granules are indicated in table 1.

The mechanisms involved in platelet degranulation are complex and have been the subject of several studies **(Holmsen, 1980).**

In vitro it is clear that some physiological stimuli such as thrombin and collagen can directly induce the release of constituents from all three granule types whereas release induced by ADP is restricted to dense bodies and alpha-granule constituents and is dependent on cell-cell contact (aggregation) and on the formation of thromboxane A_2 or the cyclic endoperoxides (PGG_2,PGH_2).

Exocytosis by platelets is similar to that in other cells in that the granule membrane fuses with the plasma membrane thereby releasing its contents into the external environment.

The invaginations of the open canalicular system may facilitate

exocytosis by effectively bringing the extracellular environment (surface membrane) into close contact with the centre of the platelet and the storage organelles located there.

2.2.5. METABOLITES OF ARACHIDONIC ACID.

This topic is discussed in more detail in section 3.1. dealing with arachidonic acid metabolism. Platelet stimulation results in the mobilization and metabolism of arachidonic acid. The free acid may be metabolised by two enzymes. Cyclooxygenase catalyses the formation of the labile intermediates the endoperoxides PGG_2 & PGH_2 which are the substrates for thromboxane synthetase, producing the even more labile thromboxane A_2 (TXA_2), and isomerases, resulting in the formation of the stable prostaglandins PGD_2 and PGE_2. Alternatively the free acid may be converted by a lipoxygenase enzyme to 12-hydroxy-eicosatetraenoic acid (HETE). Arachidonic acid metabolites are not actively secreted but, by virtue of their lipophilicity, diffuse down their concentration gradient out of the cell.

2.2.6. AGGREGATION

In addition to adhesion to collagen the development of adhesive properties on the plasma membrane also leads to platelet-platelet cohesion, provided that the cells are brought into close contact, resulting in a multicellular aggregate. In vivo aggregated platelets form the primary haemostatic plug at the site of vascular injury. In the absence of an apparent physiological stimulus platelet aggregates are responsible for various thrombotic conditions.

The mechanisms involved in platelet-platelet cohesion are distinct from those involved in adhesion to collagen. This reaction is usually but not always preceded by shape change; pseudopodia greatly enhance the chance of cell-cell contact. Since aggregation does not occur in normal quiescent cells it is probable that platelet activation results in the exposure or at least full expression of an "aggregation receptor" on the plasma membrane. A polyvalent extracellular ligand is thought to form a bridge between the "aggregation receptors" of adjacent platelets.

Considerable evidence indicates that the plasma membrane glycoproteins IIb and III are particularly important in platelet-platelet cohesion **(Nurden & Caen, 1975; Phillips & Agin, 1977).** Thrombasthenia is an inherited bleeding disorder. Platelets from patients with this condition respond normally to physiological stimuli but fail to aggregate and do

not exhibit clot retraction. **Nurden & Caen (1974)** first reported that thrombasthenic platelets were deficient in certain membrane glycoproteins and subsequent studies by **Phillips and Agin (1977)** revealed the identity of these glycoproteins as being IIb and III. **Phillips et. al. (1981)** have isolated an "aggregation complex" consisting of the cytoskeletons from individual platelets linked together in aggregates.

What is the aggregation receptor ? These workers found that glycoproteins IIb and III were the only membrane glycoproteins selectively retained with the aggregated cytoskeletons. In addition antiserum with antigenic activity towards glycoprotein IIb inhibits platelet aggregation **(Degos et. al., 1975).**

What is the nature of the intraplatelet bridge ?

The structure of the intraplatelet bridge has yet to be conclusively established. However fibrinogen, which is present in plasma, is bound to the platelet membrane, is stored at high concentrations in the platelet alpha granules and has the potential to act as a polyvalent ligand, appears to be an essential component of the bridge.

The importance of fibrinogen is emphasised by the following observations **(Berndt & Phillips, 1981):-**

1) Thrombin stimulated platelets enhances the agglutination of fixed bovine erythrocytes; the enhanced agglutination

was due to additional "agglutinin" secreted from activated platelets.

2) Various inhibitors of agglutination also inhibit aggregation.

3) Human platelet fibrinogen, treated with alpha-thrombin, induces agglutination of fixed bovine erythrocytes.

4) Platelets obtained from an afibrinogenemic donor do not secrete "agglutinin".

5) Antifibrinogen antibodies inhibit aggregation.

6) Quiescent platelets have a low affinity for fibrinogen whereas activated platelets have a high affinity and the binding requires extracellular calcium; this high affinity binding is not observed when thrombasthenic platelets are used.

It has also been demonstrated that fibrinogen itself acts as a receptor for an endogenous lectin secreted by activated platelets **(Gartner et. al., 1981)**. In a recent study a number of alpha granule proteins were examined for potential lectin activity **(Jaffe et. al. 1981)** by measuring their ability to induce agglutination of either fixed erythrocytes or platelets similar to the method described above. One particular glycoprotein thrombospondin, which is also present, in plasma was identified as having specific lectin activity. Purified thrombospondin displayed Ca++-dependent binding to platelets and induced agglutination of erythrocytes and platelets. Moreover agents that block thrombin-induced platelet agglutination also block agglutination induced by

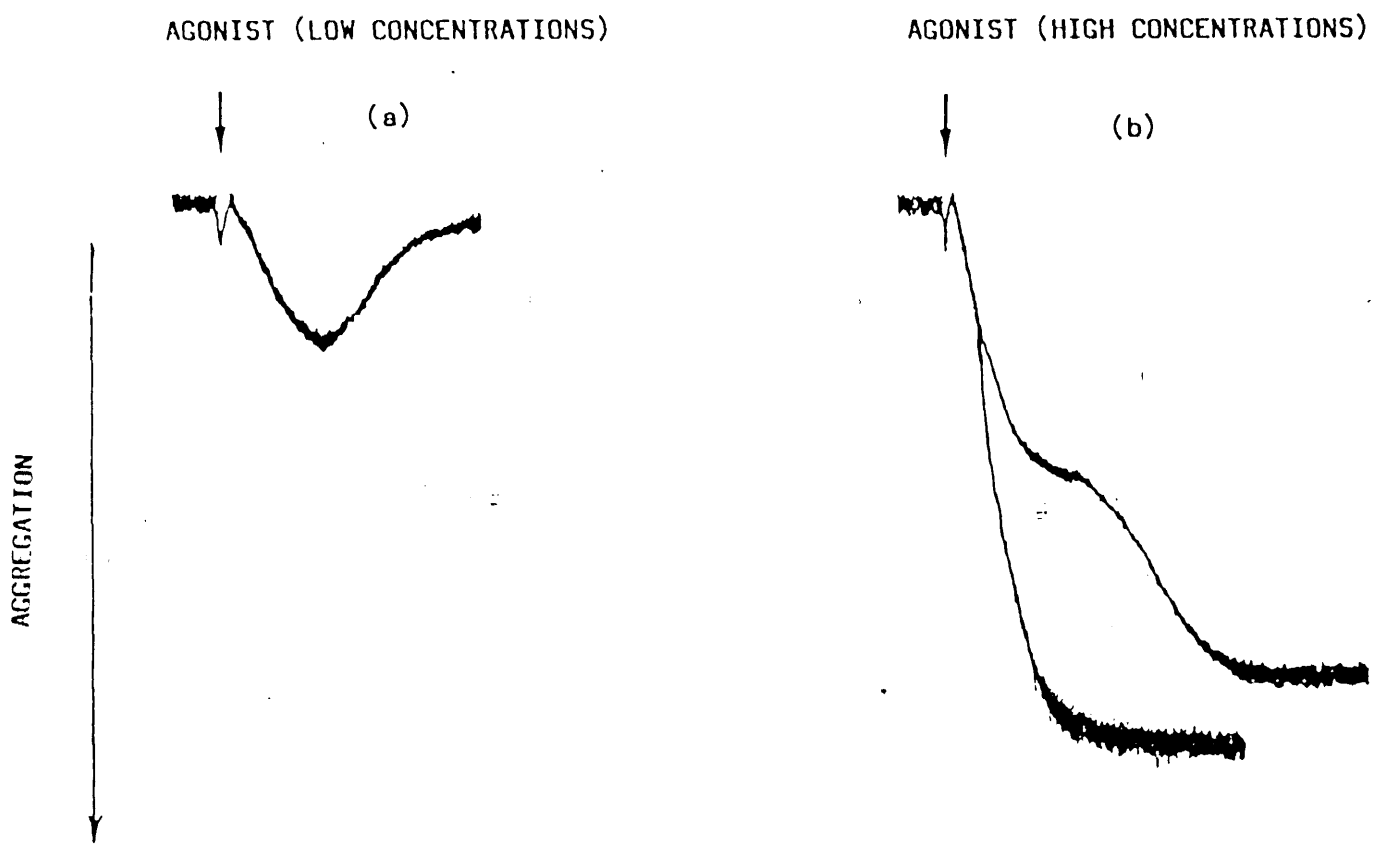


FIGURE 2. **Platelet aggregation responses measured in vitro.** a) a monophasic reversible response "primary aggregation" produced by low agonist concentrations. b) monophasic and biphasic irreversible responses "secondary aggregation" produced by high agonist concentrations.

thrombospondin. In addition excess fibrinogen inhibits thrombospondin-induced agglutination; presumably, by binding to thrombospondin, fibrinogen prevented thrombospondin binding to platelet receptor (ie.platelet bound fibrinogen).

Hence the development of platelet-platelet cohesion requires an "aggregation receptor" on the plasma membrane. This may be the glycoprotein IIb/III complex, together with a polyvalent bridging ligand in which fibrinogen, ionic calcium and a lectin, which may be thrombospondin, are essential components.

Gartner (1979) has suggested that several different platelet-platelet bridges may occur. In support of this view it is known that thrombasthenic platelets, which lack glycoprotein IIb/III, do not display mutual cohesion but cohere with normal platelets, indicating that, at least in this situation, an asymetrical bridge can exist; ie. different bonds are important on opposite platelets **(Gerrard et. al., 1979).**

2.2.6a. PLATELET AGGREGATION: "PRIMARY" AND "SECONDARY" AGGREGATION.

Platelet activation can be monitored in vitro. The most common technique is the photometric measurement of platelet aggregation by the method developed by **Born (1962).** When aggregation is monitored in this way two distinct responses are observed, "primary" aggregation and "secondary" aggregation (figure 2).

"Primary" aggregation is a monophasic reversible response not associated with secretion of platelet granule constituents nor

accompanied by arachidonic acid metabolism. **(Mustard & Packham 1970)**

"Secondary" aggregation can be monophasic or biphasic and is associated with secretion of granule constituents, arachidonic acid mobilization and the biosynthesis of prostaglandin endoperoxides (PGG_2 & PGH_2) and thromboxane A_2 (TXA_2). That is, the observed response is initiated by the exogenous agonist but is propagated by endogenous agents: PGG_2, PGH_2, TXA_2, secreted ADP and 5HT, by inducing further platelet aggregation, provide a mechanism for positive feedback **(Holmsen et. al., 1969; Hamberg et. al., 1975)**

In addition to TXA_2, PGG_2 and PGH_2 platelets have the potential to synthesise other proaggregatory lipids: platelet activating factor (PAF) and lysophosphatidic acid (LPA) which may also be potentially important mediators of platelet activation. The involvement of lipids in mediating platelet reactivity is discussed in section 4.

Presumably both exogenous and endogenous agonists act on specific receptors on the plasma membrane or perhaps in the case of lipophilic agents at the cell interior. In platelets, as in other cells, the concentration of free calcium is thought to be the principle intermediary that links receptor occupancy to functional response **(Gerrard, 1981)**. The role of calcium and the ability of the cell to modulate its functional response by regulating the concentration of cytosolic free calcium is discussed in section 3.

2.3. PATHO-PHYSIOLOGICAL ROLES OF PLATELET ACTIVATION.

2.3.1. ROLE OF SECRETION IN HAEMOSTASIS.

In vivo many of the platelet responses involved in the haemostatic process occur simultaneously therefore it is difficult to demonstrate the inter-relationship of secretion with other platelet responses. Histologically the initial step in the haemostatic process is the adhesion of circulating platelets to subendothelial collagen. Collagen, a powerful secretagogue, induces secretion by these adherent cells; a process thought to be responsible for propagating the haemostatic response. Specifically stimulation of platelets in the immediate vicinity of the adherent cells results in aggregation of platelets and the formation of a platelet aggregate. The secreted agents responsible for propagation are the arachidonate metabolites TXA_2, the endoperoxides PGG_2 & PGH_2, ADP and 5HT.

TXA_2 is also a potent vasoconstrictor, constriction of the damaged blood vessel may be an important mechanism in the cessation of bleeding.

Thrombin formation is also important in propagating the haemostatic response. Activated platelets, by exposing PF3, not only promote thrombin formation but secrete agents which prevent it's inactivation. Several alpha-granule proteins PF4, LAPF4, BTG, PDGF and fibronectin display heparin neutralising activity, of these PF4 has the highest activity **(Niewiarowski & Paul, 1981).**

2.3.2. CLOT RETRACTION.

This is the process whereby the loose meshwork of platelets and fibrin, which make up the haemostatic plug, condenses to form a clot. This response is unaffected by colchicine treatment and it is generally accepted that the contractile force underlying the event is provided by actomyosin. One model, proposed by **Cohens & de Vries (1973)** suggests that the extensive bundles of actin filaments, present in the pseudopodia, in association with myosin act against the inelastic fibrin strands of the plug.

The discovery that the glycoprotein complex IIb/III is retained on the actin filaments of aggregated platelets **(Phillips et. al., 1983)** support this model since these glycoproteins are probably the binding site for fibrinogen **(Berndt & Phillips, 1981)** and may serve to connect fibrin strands on the outer surface of the platelet to the actin filaments.

2.3.3. INFLAMMATION.

Platelets that accumulate at the site of vascular injury produce an increase in vascular permeability **(Mustard et. al., 1965). Nachman et. al. (1972)** reported that a cationic protein (Mwt. 30,000 ; permeability factor) caused an increase in vascular permeability by inducing histamine release from mast cells. This same protein also acts on the fifth

component of complement (C5) to liberate a fragment with chemotactic activity for polymorphonuclear (PMN) leukocytes **(Packham, 1968)**; leukocytes secrete potent permeabilizing agents. Certain arachidonate metabolites also share these activities. **Silver et. al. (1974)** reported that PGE2 directly increased vascular permeability, while **Turner et. al. (1975)** demonstrated that HETE is also chemotactic for PMN leukocytes. In addition released 5HT has a profound influence on vascular permeability **(Gordon & Milner, 1976).**

2.3.4. WOUND HEALING.

Some of the most potent serum mitogens are proteins secreted from platelet alpha-granules and, in vitro, stimulate the proliferation of several cell types including fibroblasts and smooth muscle cells **(Ross, 1974; Rutherford & Ross, 1976; Kohler & Lipton, 1974).** The platelet proteins which show mitogenic activity are PBP, PDGF and LAPF4. PBP and PDGF appear to possess similar mitogenic activity **(Heldin et. al., 1979).** LAPF4 is immunologically identical with PBP but is a weaker mitogen **(Paul et. al., 1980).**

2.3.5. ATHEROGENESIS.

Migration of smooth muscle cells from the media to the intima and subsequent proliferation causing intimal thickening may be due to factors released from adherent platelets that are both chemotactic and mitogenic (eg. PDGF). In addition it is known

that persistent mural thrombi are eventually covered by endothelium and incorporated into the arterial wall leading to further vessel occlusion **(Mustard & Packham, 1975).**

3. MECHANISMS THAT REGULATE PLATELET REACTIVITY.

3.1. CALCIUM AND CELL REGULATION

Inorganic ions, especially divalent metal cations, are essential cofactors in many biological reactions. By regulating the intracellular concentration of these ions, through selective permeability changes in the plasma membrane, cells can control the intracellular reactions responsible for energy metabolism, exocytosis, muscle contraction and other cellular responses.

A central role for calcium, as the principle divalent metal ion for regulation of cell function, was advocated and pioneered by L.V. Heilbrumn in the earler half of this century. Such a role was initially regarded with scepticism and only in recent years, with the development of techniques for manipulating calcium concentrations, has this role gained wide acceptance.

The cytosolic concentration of free calcium ($[Ca^{++}]_i$) in the cell is maintained at very low levels relative to the extracellular concentration, typically 10^{-7}M. To achieve this large concentration gradient cells actively transport calcium out across the plasma membrane by means of an ATP-dependent Ca++-pump or Ca++/Na+ exchange. Alternatively certain intracellular organelles, such as mitochondria and reticulum, are able to sequester calcium.

In maintaining a low Ca++ the cell has the ability to regulate all Ca++-dependent events simply by increasing $[Ca^{++}]_i$. Consequently, in response to a physiological stimulus, a transient increase in Ca++ permeability of the plasma membrane or in some cases translocation of intracellular calcium is

evoked resulting in an elevation of $[Ca^{++}]_i$ and stimulation of calcium-dependent reactions. Calcium therefore, may be considered as a biochemical intermediary or "second messenger" in stimulus-response coupling.

In order to act as an intermediary or second messenger calcium itself must act on a receptor. There appears to exist specific calcium receptor proteins, notably calmodulin and troponin, which confer calcium sensitivity to many of the biochemical reactions underlying cellular responsiveness.

3.2. THE ROLE OF CALCIUM IN PLATELET ACTIVATION.

The first indication that an elevation in $[Ca^{++}]_i$ may regulate platelet functional response came from observations using ionophores for divalent cations; A23187 induces shape change, aggregation and secretion in platelet suspensions **(Feinman & Detwiler, 1974; Massini & Luscher, 1974; White et. al., 1974; Feinstein & Fraser, 1975).** These antibiotics effectively increase membrane permeability for divalent ions. Consequently A23187 would allow extracellular calcium to pass, down its concentration gradient, into the cytoplasm hence increasing $[Ca^{++}]_i$. Indeed A23187 can induce a net uptake of $^{45}Ca^{++}$ **(Massini & Luscher, 1974).** In addition TMB-8 an intracellular calcium antagonist in muscle **(Malagodi & Chiou, 1975)** inhibits platelet secretion induced by A23187 which can be overcome by increasing extracellular calcium **(Charo et al, 1976).**

In the absence of extracellular calcium aggregation is

abolished. Thus extracellular calcium is essential for aggregation and is thought to act by stabilizing intra-platelet bridges **(Gerrard, 1981)**. However in human platelets shape change and secretion are relatively unaffected under these conditions. It is suggested that these responses are mediated by translocation of calcium from intracellular storage pools to the cytosol.

Intracellular calcium in platelets is distributed in several storage sites. The major site is the dense granules, containing approximately 60% of all platelet calcium. The mechanism by which calcium is transported into this organelle is unknown and although dense granules constitute the largest calcium pool in platelets, this pool does not appear to contribute to $[Ca^{++}]_i$. **(Gerrard, 1981)** The dense tubular system (DTS) (a form of reticulum) has been compared to sarcoplasmic reticulum, a well defined calcium-accumulating organelle in skeletal and cardic muscle, and may well perform a similar function in platelets **(Statland et. al., 1969)**. Indeed a membrane fraction containing Ca^{++}-dependent ATPase activity, and thought to be DTS, has been isolated from platelets **(Kaser-Glanzman et. al., 1977)** In addition to the DTS platelets also contain mitochondria and, while the calcium accumulating characteristics are not well known, by analogy with other cells, platelet mitochondria may also function as a calcium storage organelle **(Scharf & Luscher, 1979)**.

That platelets can utilize $[Ca^{++}]_i$ is supported by the observation that platelet activation induced by ADP is inhibited by deuterium oxide (D_2O) **(Le Breton et. al.,**

1976). D_2O inhibits the release of calcium from sarcoplasmic reticulum in muscle **(Kaminer et. al., 1972).** Since the DTS is thought to function in a manner analogous to sarcoplasmic reticulum it seems likely that the inhibition of platelet function by D_2O is mediated by blocking calcium release from the DTS.

The origin of $[Ca^{++}]_i$ has been the subject of many investigations. Ionophore studies indicate that platelets can utilise both extracellular calcium and calcium translocated from intracellular storage pools. The relevent importance of each of these sources, in a physiological context, is the subject of much debate and may in fact depend on the stimulus **(Le Breton, 1982).**

While studies with ionophores indicates the importance of calcium in platelet activation the evidence for a central role is circumstantial. Direct evidence that an elevation in $[Ca^{++}]_i$ mediates platelet activation has been reported by **Le Breton et. al. (1982).** Using the fluorescent calcium probe, chlortetracycline (CTC) they demonstrated that A23187 and more importantly the physiological stimulus ADP and U44069 (a TXA_2 mimetic) induce an elevation in $[Ca^{++}]_i$, further, a rise in $[Ca^{++}]_i$ could also be evoked in conditions of low extracellular calcium, presumably due to calcium translocation. Also using this technique **Feinstein (1980)** has shown that the elevation in $[Ca^{++}]_i$, induced by thrombin, precedes dense body secretion.

These results apparently demonstrate the central role of $[Ca^{++}]_i$

in platelet activation. However changes in CTC-fluorescence are not conclusive since its precise location within the platelet is unknown and could partition into calcium containing organelles. In addition it is not absolutely clear that the changes are specific for calcium since magnesium ions also have a high affinity for CTC **(Caswell, 1972).**

In the "shocked cell" technique small pores are produced in the plasma membrane by means of a localized electrical discharge. This allows passage of small ions between the extracellular medium and the cytosol. Manipulation of extracellular calcium allows $[Ca^{++}]_i$ to be varied in a controlled and quantifiable manner. Using this techique **Knight et. al. (1982)** have titrated $[Ca^{++}]_i$ required to evoke lysosomal enzyme release and secretion of dense body constituents. They found that half maximum release, for both granule types, occurred when $[Ca^{++}]_i = [Ca^{++}]_o = 1.9\ \mu M$.

The most compelling evidence for the role of calcium has been demonstrated using the fluorescent calcium indicator dye Quin2 **(Tsien, 1982).** This dye has a high affinity for ionic calcium, is located predominatly in the cell cytoplasm and unlike CTC, which measures relative changes in $[Ca^{++}]_i$, quantifiable changes in $[Ca^{++}]_i$ may be made (see results). Using the calcium ionophore ionomycin **Rink et. al. (1982)** have also titrated the $[Ca^{++}]_i$ threshold required to evoke various functional responses. They found that shape change was induced at $[Ca^{++}]_i$ = 400 - 600 nM, release of dense body constituents occured at $[Ca^{++}]_i$ = 0.7 - 1 μM and aggregation required $[Ca^{++}]_i$ = 2 μM.

From the results obtained in this study it is clear that the concentrations of physiological stimuli that evoke platelet functional responses do not elevate $[Ca^{++}]_i$ to the values titrated by Rink or Knight. This being the case Ca++ alone may not be the only second messenger in platelets. Such a view is supported by the observation that collagen, a potent aggregating agent and secretagogue, does not appear to elevate $[Ca^{++}]_i$ **(Rink et. al. 1983).**

In addition to elevating $[Ca^{++}]_i$ most platelet agonists, including collagen, induce inositol phospholipid hydrolysis. Diacylglycerol (DAG), a transient intermediary of this hydrolysis, may represent a Ca++-independent second messenger **(Nishizuka, 1984).**

DAG activates a protein kinase, protein kinase C, (see section 3.3.4.) an event that occurs normally during stimulation and is mimicked by phorbol esters (12-O-tetradecanoyl phorbol-13-acetate) (TPA). In platelets exogenous diacylglycerol (1-oleoyl-2-acetyl-glycerol) (OAG) or TPA do not elevate $[Ca^{++}]_i$, yet evoke secretion and aggregation in a manner resembling that produced by collagen **(Rink, 1983).** Indeed **Rink et. al. (1983)** have demonstrated that a small sub-threshold elevation in $[Ca^{++}]_i$, induced by ionomycin, synergises with OAG or TPA to produce secretion similar to that induced by physiological stimuli.

It is known that several platelet agonists, including ADP and adrenaline, can inhibit adenylate cyclase and since an elevation in cAMP is associated with platelet inhibition,

Salzman (1974) has proposed that the converse, inhibition of adenylate cyclase, may be a possible mechanism for activation. However **Haslam et. al. (1978)** has reported that inhibition of adenylate cyclase does not induce or potentiate aggregation.

3.3. INTER-RELATIONSHIP BETWEEN CALCIUM AND CYCLIC NUCLEOTIDES.

While calcium appears to be one of the second messengers that links receptor occupancy to functional response cAMP represents the most important inhibitory second messenger. cGMP also exerts inhibitory effects on platelet function. As the effects Ca++ and cAMP/cGMP are antagonistic in platelets, it might be expected that they exhibit an inverse relationship: that is an elevation in one inhibits the availability of the other.

Much of the experimental evidence on this topic indicates that cAMP can inhibit the availability of Ca++. For example **Kaser-Glanzmann et. al. (1977)** have demonstrated that the Ca++-accumulating activity of isolated membrane fractions is markedly enhanced in the presence of cAMP. In intact platelets **Owen and Le Breton (1981)** have shown that Ca++ binding is inversely related to intracellular levels of cAMP. In addition phosphodiesterase inhibition augments Ca++ binding **(Owen and Le Breton, 1981)**

On the other hand **Roden and Feinstein (1976)** have demonstrated that Ca++ is a potent inhibitor of adenylate cyclase. It has also been shown that TMB-8 diminishes the ability of TXA_2 to inhibit adenylate cyclase **(Gorman et. al., 1979)** and that platelet inhibition, due to cAMP, may be overcome by high concentrations of the Ca++ ionophore A23187 **(Le Breton et. al., 1982)**. Presumably the ionophore maintains a rate of influx/release which is greater than the rate of extrusion and/or resequestration promoted by cAMP, resulting in a net elevation in $[Ca^{++}]_i$. Hence it would appear

that a bidirectional relationship exists between Ca++ and cAMP. The effects of cGMP on Ca++ availability in platelets have apparently not been examined. However evidence in other systems (**Schultz, 1977**) would suggest that cGMP may act to regulate Ca++ availability (discussed in section 9). Similarly the effect of Ca++ on platelet guanylate cyclase has not been investigated.

4. PUTATIVE LIPID MEDIATORS OF PLATELET ACTIVATION

Various classes of lipids can induce platelet activation which range from fatty acids (icosanoids), to ether lipids (PAF), to phospholipids (PA, LPA) and to neutral lipids (DAG).

4.1. PLATELET ARACHIDONIC ACID.

Arachidonic acid (AA) (5,8,11,14,-eicosatetraenoic acid) is one of the most abundant fatty acid in platelets. It is derived either from modification of the essential fatty acid linoleic acid (obtained from a vegetable diet) or directly from a diet containing animal products. The free acid is present only in trace quantities in the cytosol, being incorporated into lyso-phosphatides by an acetyl CoA synthetase and acyltransferase(s) - the activities of which are probably reflected by the low background level of AA. Virtually all arachidonic acid is esterified in the sn 2 position of phospholipids. Phosphatidylethanolamine (PE) contains most of the arachidonic acid 49 - 55 % with 26 - 31 % contained in phosphatidylcholine (PC) and the remaining 14 - 25 % distributed between phosphatidylserine (PS) and phosphatidylinositol (PI) **(Marcus et. al., 1969; Cohen & Derksen, 1969).**

4.1.1. MOBILIZATION OF ESTERIFIED ARACHIDONIC ACID

Icosanoids, the metabolic products of arachidonic acid, are not stored in platelets but are synthesised during platelet

activation. Only the free acid may serve as a substrate for metabolizing enzymes. Hence the first step in arachidonic acid metabolism is its release from membrane phospholipids. The phospholipid substrate and mechanism by which AA is liberated is the subject of much contention. The reason for such contention becomes apparent from the following observations. The total content of AA in platelets is around 100 nmol/10^9 cells **(Neufeld & Majerus, 1983)**. Activated platelets have been reported to be capable of releasing 15 - 25 % of the total AA in less than 30 sec. **(Majerus, et. al., 1983; Smith, et. al., 1985)**. The free acid is rapidly metabolised by cyclooxygenase and lipoxygenase; over 60% in 2 min. **(Hamberg & Samuelsson, 1974)**, and to a much smaller extent by isomerases. In addition acyltransferase activity is likely to reincorporate free acid and is probably the mechanism underlying the observation that AA is transfered to plasmalogen PE following activation **(Rittenhouse-Simmons et al., 1981)**. It is these factors that make an estimation of the amount of free acid liberated, its phospholipid origin and mechanism(s) by which it is liberated particularly difficult to determine. **Bills et. al. (1977)** first demonstrated that radiolabelled AA was released from the sn-2 position of phospholipids in much greater proportions than other fatty acids and proposed that the selectivity was due to the action of a phospholipase A_2 (PLA_2) enzyme. PLA_2 enzymes have been extracted and purified from platelets in several studies **(Derksen & Cohen, 1975; Jesse & Cohen, 1976; Jesse & Franson, 1979; Apitz-Castro et. al., 1979)**. Additional support for such a mechanism comes

from observations by **Broekman et. al. (1980)** that, in response to platelet agonists, 1-acyl-sn2-lyso phospholipids are formed in parallel with the free acid.

In studies devised to determine the phospholipid origin of AA, in platelets prelabelled with radioactive AA, were reported to lose their label principally from PC and PI **(Bills et. al., 1976; Russel & Deykin, 1976; Schoene, 1978).** For example, in a study using rabbit platelets 41 % of the label came from PC with 28 % from PI, 22 % from PE and 7 % from PS **(Van Den Bosch, 1980).** However these initial studies did not take into account the size of the phospholipid pools, that is the distribution of AA within the various phospholipids species. When this factor (pool size) was considered the contribution made by PC was estimated to be 14 %, PI = 24 % and PS = 3 % whereas PE contributed the majority of free acid, approximately 58 % **(Van Den Bosch, 1980).**

Indeed, in human platelets, when lyso-phospholipids were quantitatively monitored **Broekman et. al. (1980)** reported a rapid hydrolysis of PI, lyso PE consisting of both 1-alk-1 enyl and 1-acyl forms were observed as well as a small rise in lyso-PC but only after 60 sec. No change in PS was observed. Hence by acting as a substrate for PLA_2 PE, PC and PI may serve as a source of AA in platelets.

In experiments using disrupted platelets **Bell, et. al. (1979)** were unable to detect sufficient PLA_2 activity to account for the amount of AA released by intact platelets following supramaximum stimulation. In addition, the PLA_2 activity found was not specific for 2-arachidonyl- and acted

equally on 2-oleoyl- phospholipids.These workers have therefore proposed an alternative pathway to account for the selective release of AA. In this scheme 1,2-diacylglycerol (DAG) is formed by the action of a PI specific PLC enzyme (see section 3.3.1. for details), AA is then cleaved from DAG by a DAG-lipase. As PI hydrolysis is one of the earliest biochemical events initiated by platelet agonists and as DAG, formed transiently, is rich in AA this scheme represents a plausible mechanism for the liberation of AA. Moreover sufficient DAG lipase activity has been extracted from platelets to support such a mechanism **(Bell et. al., 1979).**

These workers have estimated that the amount of AA released by platelets, in response to supramaximum concentrations of agonists , to be approximately 10 nmol/10^9cells/min. They calculate that 5 - 10 nmol/10^9cells of PI is hydrolysed within 10 - 60 sec. and hence could account for all the AA released by the intact cell. Critics argue, however, that the bulk of PI hydrolysed is recycled back to PI via phosphatidic acid (PA) **(Lapetina, 1982)** hence the majority of DAG would be phosphorylated to PA. Indeed, in platelets, the level of PA rises following activation **(Lapetina et al., 1979).** However, it has been pointed out that the fatty acids in resynthesised PI may differ from those in the origional PI **(Imai et. al., 1981; Prescott & Majerus, 1981)** suggesting at least some of the PA recycled to PI originates from synthesis de novo.

In a more recent study **Smith et. al. (1985)** have shown that in the presence of both CO and LO inhibitors (LO inhibition was

absent in the study by Bell and colleagues) that 32 nmol AA/10^9 cells could be released following treatment with high concentrations of thrombin, representing approximately 25 % of the total platelet AA. In this study Smith demonstrated that 40 % of the PI hydrolysed is recovered as 1-stearoyl-2-arachidonoyl PA. Hence only 60 % of the PI hydrolysed could be utilised by the DAG lipase pathway which could only account for > 15 % of the AA released in this study. Further studies by **Chau and Tai (1983)** using platelet membranes have suggested that the release of AA via this pathway requires the sequential action of a diglyceride lipase and a monoglyceride lipase. The DG lipase catalyses the deacylation of DG at the sn-1 position thereafter AA is cleaved by a monoglyceride lipase. In support of this pathway for liberation of AA this group have shown that there is a transient accumulation of arachidonyl monoglyceride as well as arachidonyl diglyceride in prelabelled human platelets following thrombin treatment. They also found that addition of a specific DG lipase inhibitor abolished the accumulation of monoglyceride but not of AA, suggesting that, while this pathway could contribute to the liberation of AA, its inhibition does not markedly alter agonist-induced AA release. It is possible, as suggested by Lagarde **(Guichardant & Lagarde, 1980)**, that the PLC/DG Lipase pathway may play a more significant role when submaximal stimuli are employed.

A further mechanism by which AA may be liberated again necessitates the hydrolysis of PI. **Lapetina (1982)** has

described a PLA_2 enzyme which favours the specific degradation of PA. **Lapetina (1982)** argues that the agonist-induced accumulation of PA, in platelets, preceeds the accumulation of AA and therefore could act as a source for the latter. However other workers have shown that the accumulation of PA parallels that of AA in platelets **(Majerus et. al., 1983)** and in some tissues lags behind the physiological response **(Cockcroft et. al., 1980; Farese et. al., 1981)** (discussed in section 4.3.3.). One would expect that with such a mechanism lyso PA would be formed in amounts equivalent to the free acid, however the amount of lyso PA recovered after platelet stimulation represents about 10 % of the accumulated PA **(Lapetina et. al., 1981)** which would suggest that only a fraction of PA formed is hydrolysed to sn-2 lyso PA. However Lapetina argues that rapid reacylation of lyso-PA occurs by a unique transferase which transfers AA from PE and PC (discussed in section 3.3.5.). Such an enzyme has been demonstrated in rat liver microsomes **(Irvin & Dawson 1979)**, and a third type of PLA_2 enzyme would presumably be an integral component of this system. The origi nal experiments by Lapetina and colleagues were conducted on horse platelets, since then attempts to demonstrate the existence of a PA specific PLA_2 enzyme in human platelets have failed. It has also been argued that the lyso PA formed upon thrombin stimulation is 1-lyso-2-acyl glycerol -3-phosphate which indicates a PLA_1 activity rather than PLA_2 **(Mauco et. al. 1978).**

The relative importance of the various pathways leading to liberation of AA remains to be assessed fully. However at

present degradation of phospholipids by PLA_2 appears quantitatively to be the most important mechanism.

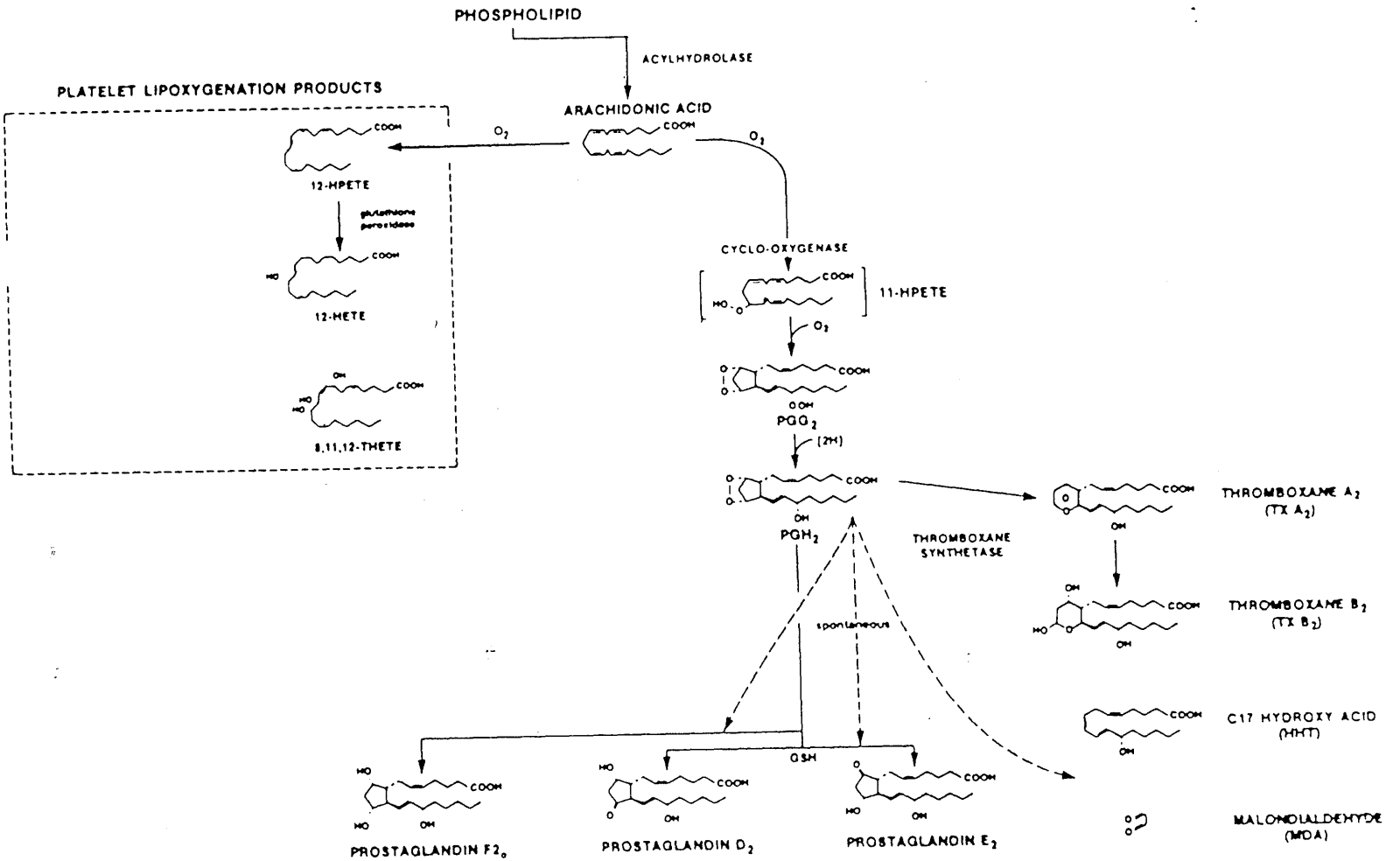


Figure 3. Metabolic pathways of arachidonic acid in platelets.

4.1.2. METABOLISM : THE CYCLOOXYGENASE PATHWAY. (Fig. 3)

Cyclooxygenase rapidly catalyses the formation of cyclic endoperoxides PGG_2 and PGH_2 from free arachidonic acid **(Hamberg & Samuelsson, 1973, 1974).** This enzyme consists of two components, the initial oxygenation of arachidonic acid , giving rise to the so called " oxygen burst " or " respiratory burst " is catalysed by prostaglandin endoperoxide synthetase resulting in the formation of PGG_2. Subsequent reduction of PGG_2, yeilding PGH_2, is catalysed by cyclooxygenase peroxidase. Both PGG_2 and PGH_2 are short lived (T 1/2 = 5 min. at 37°C) intermediates and are converted principally into thromboxane A_2 (TXA_2) by the enzyme thromboxane synthetase **(Smith et. al., 1976; Hamberg et. al., 1975).** TXA_2 is extremely labile with a half life of 30 sec. in aqueous medium and approximately 5 min. in plasma,: the increased stability being due to binding to plasma albumin. Its formation is normally detected by the appearance of its stable breakdown product thromboxane B_2 (TXB_2).

In addition to thromboxane formation platelet isomerase enzymes also catalyse the formation of small amounts of stable icosanoids (PGE_2, $PGF_{2\alpha}$ and PGD_2) from the cyclic endoperoxides . The total production of these stable prostaglandins amount to less than 1 % of the amount of TXB_2 synthesised **(Smith et. al., 1973).** Two other stable products are formed in equimolar amounts with TXB_2 these are:- 12-hydroxy-5,8,10 heptadecatrienoic acid (HHT) and malondialdehyde (MDA).

4.1.3. EFFECTS OF CYCLOOXYGENASE METABOLITES ON PLATELET REACTIVITY.

4.1.3a. THROMBOXANE A_2 AND THE ENDOPEROXIDES

The first action of the arachidonate metabolites on platelets was reported by **Willis & Kuhn (1973)** and **Vargaftig & Zirinis (1973),** and the active moiety was termed "Labile Aggregating Stimulating Substance" (LASS) by the former group, reflecting it's major action - platelet aggregation and degranulation. A vasoconstrictor activity had been reported by **Piper & Vane (1969)** who called it "Rabbit Aorta Contracting Substance" (RCS). Although TXA_2, the principle constituent of LASS and RCS, has been reported to possess calcium ionophore activity **(Gerrard et. al., 1978).** It's platelet aggregating and vasoconstrictor activity are almost certainly mediated by combination with specific receptors **(MacIntyre, 1981).**

Early observations indicated that the formation of TXA_2 was a prerequisite for platelet aggregation **(Hamberg et. al., 1975).** However it is now clear that the endoperoxides PGG_2 & PGH_2 can directly induce aggregation and are slightly less potent than TXA_2 (in platelets) **(Hamberg et. al., 1975; Moncada & Vane, 1977)** and may in fact share the same receptor **(Le Breton 1983; Jones, 1984).** The importance of these agents in the haemostatic response has been discussed under platelet secretion.

In vitro platelet stimulation by certain agonists such as PAF,

ADP, adrenaline and low, but not high, concentrations of thrombin and collagen have an absolute requirement for TXA_2 or PGG_2/PGH_2 to evoke degranulation. The mechanism underlying this action of $TXA_2/PGG_2/PGH_2$ is unknown.

4.1.3b. OTHER PROSTANOIDS

The naturally occuring prostaglandins (TXA_2/PGG_2,PGH_2, PGE, PGF, PGI, PGD) exhibit a unique biological profile. In general, studies using techniques such as rank order of agonist potency **(Coleman et. al., 1980; Jones, 1984)** selective antagonists **(MacIntyre & Salzman 1978)** and binding studies **(Hung et. al., 1982; Siegl et. al., 1979)** indicate that a specific receptor exists for each of the natural prostaglandin classes, with a possible subclassification of the PGE_2 receptor **(Jones, 1984).** In platelets initial studies identified and characterised stimulatory and inhibitory receptors **(MacIntyre & Salzman 1978).** Since then most studies have concentrated on characterising the TXA_2/PGH_2 and PGI_2 receptor. Much less information is known about the PGD_2, $PGF_{2\alpha}$ and PGE_2 receptors however it is likely that these prostaglandins also act by combining with distinct receptors on the platelet. Indeed the original experiments by **MacIntyre and Salzmann (1978)** using selective antagonists identified a distinct PGD_2 receptor and more recently binding studies have identified a single PGD_2 receptor type with a Ka of 4.1 10^{-7} M and approximately 600 binding sites per platelet

(Siegl et. al., 1979). Exogenous PGD_2 inhibits platelet functional responses. The concentrations of PGD_2 which produce inhibition are associated with an elevation in platelet cAMP **(Smith et. al. 1974).** Hence the inhibitory effect of this prostaglandin in platelets apparently is mediated by combination with a unique receptor coupled to adenylate cyclase.

Two effects have been reported for PGE_2. Low concentrations of this prostaglandin have been reported to enhance agonist-induced aggregation, particularly "secondary aggregation" induced by ADP, while higher concentrations inhibit agonist-induced platelet functional responses **(Kloeze 1967; Shio & Ramwell 1972).** The concentrations of PGE_2 which produce inhibition are associated with an elevated level of platelet cAMP **(Salzman et al., 1972; McDonald & Stuart 1974).** In theory a sub-population of the PGE_2 receptors could explain the dual effects of exogenous PGE_2. Such is the case in smooth muscle where one receptor type mediates a stimulatory response and the other an inhibitory response **(Jones, 1984).** However stimulation of an excitatory receptor would be expected to enhance both primary and secondary aggregation. Low concentrations of PGE_2 have been reported to inhibit adenylate cyclase **(Salzman 1976),** again however, one would expect, as cAMP inhibits all functional responses, that inhibition of adenylate cyclase would enhance all platelet responses and not just secondary aggregation. It has been reported that the enhancement of aggregation by

PGE_2 is blocked by aspirin **(Shio & Ramwell, 1972)** suggesting that its effect is mediated by a metabolite of cyclooxygenase. One proposal which might account for this effect is that low concentrations of exogenous PGE_2, which do not activate adenylate cyclase, antagonise the effect of agonist-induced PGD_2 **(Willis & Smith 1981)**. Presumably PGE_2 binds to the PGD_2 receptor without evoking a functional response thereby preventing the action of PGD_2. It is known that the effects of exogenous PGD_2 are blocked by PGE_2 **(Anderson et. al., 1980)**. Such a hypothesis, however, assumes that during the course of a normal agonist-induced response (secondary aggregation) PGD_2 is formed in sufficient amounts to elicit a biological response. Indeed it has been suggested that PGD_2 may in fact be produced in sufficient quantities to evoke a biological response, fuelling speculation that it may be inolved in a negative feedback system which limits the extent of platelet activation **(Smith et. al. 1974)**. Though this view is not universal as some studies suggest that the amount of PGD_2, around 10pmol/ml and upwards, required to inhibit aggregation vastly exceeds that amount produced by activated platelets **(Oelz et. al., 1977)**.

$PGF_{2\alpha}$ has been reported to selectively inhibit platelet aggregation induced by arachidonic acid and the thromboxane mimetic U46619 **(Hung et. al, 1982)**. In these studies the authors were unable to relate inhibition to changes in cAMP content but demonstrated that specific $[^3H]PGF_{2\alpha}$ binding

could be displaced by both the U46619 and the TXA_2/PGH_2 receptor antagonist 13-azaprostanoic acid. It was concluded from these studies that $PGF_{2\alpha}$ interacted with the TXA_2/PGH_2 receptor.

In contrast **Armstrong and colleagues (1983)** demonstrated that $PGF_{2\alpha}$ inhibited primary aggregation induced by PAF, thrombin and the TXA_2 mimetic 11, 9-epoxymethano-PGH_2 but not ADP. These workers found that $PGF_{2\alpha}$ could elevate platelet levels of cAMP and cGMP. Inhibition of adenylate cyclase but not guanylate cyclase inhibited the effect of $PGF_{2\alpha}$. It was concluded from these studies that the action of $PGF_{2\alpha}$ was mediated via stimulation of adenylate cyclase: indeed the lack of effect on ADP would support this view as ADP can directly inhibit adenylate cyclase.

Further studies will be required to determine the exact the mode of action of $PGF_{2\alpha}$.

4.1.4. LIPOXYGENASE PATHWAY. (Fig. 3)

The other route by which arachidonic acid may be metabolised is via a lipoxygenase enzyme to HPETE (12-L-Hydroxyperoxy-5,8,10,14-eicosatetraenoic acid) and subsequently reduced to HETE (12-L-Hydroxy-5,8,10,14-eicosatetraenoic acid) **(Hamberg & Samuelsson, 1974).**

4.1.5.EFFECTS OF LIPOXYGENASES METABOLITES ON PLATELET REACTIVITY.

12-HPETE, like TXA_2, is short lived in platelets and is rapidly reduced to 12-HETE. Both 12-HPETE and 12-HETE have been shown to inhibit platelet aggregation, the hydroperoxy intermediate being approximately three fold more potent than the corresponding hydroxy derivative **(Aharony et al., 1982; Vericel & Lagarde, 1980,1981).** The mechanism underlying this inhibition has not been clearly resolved. 12-HPETE has been reported to inhibit human platelet thromboxane synthetase **(Hammarstrom & Falardeau, 1977).** However more recently **Croset and Lagarde (1983)** have shown that 12- and 15-HPETE/HETE specifically inhibit PGG_2-PGH_2/TXA_2-induced platelet aggregation without affecting their formation from exogenous arachidonic acid. The biological role of the lipoxygenase metabolites, in platelets, is unknown, however **Samuelsson et. al. (1976)**

have shown that the formation of lipoxygenase metabolites lags behind the formation of cyclooxygenase metabolites. As the former metabolites appear to be inhibitors while the latter are potent platelet activators, 12-HPETE and 12-HETE may act as part of a negative feedback system to limit the extent of activation.

4.1.6. ARACHIDONATE METABOLITES AS REGULATORS OF PLATELET ACTIVITY IN VIVO: A POSSIBLE ROLE FOR TXA_2 AND PROSTACYCLIN.

The major metabolite of endothelial cell arachidonic acid is prostacyclin (PGI_2), formed by the enzyme PGI_2-synthetase, and like TXA_2 extremely labile. PGI_2 and TXA_2 exert opposing effects: TXA_2 promotes aggregation and vasoconstriction while PGI_2 inhibits platelet aggregation and promotes vasodilatation. It has been proposed that the activity of circulating platelets and vascular homeostasis depends on the balance between TXA_2 AND PGI_2 **(Moncada & Vane, 1979)**. This balance may be disturbed in certain pathological conditions. For example high concentrations of lipid peroxides, which inhibit PGI_2-synthetase **(Moncada et. al., 1976)** are associated with atherosclerotic lesions **(Glavind et. al., 1952)**. Hence the lipid peroxides present in this pathological condition could inhibit PGI_2 in the vascular wall without impairing TXA_2 generation by platelets. In contrast a characteristic of uraemia is an increased bleeding time apparently due to a platelet defect. It has been suggested that TXA_2 formation in these platelets might be depressed.

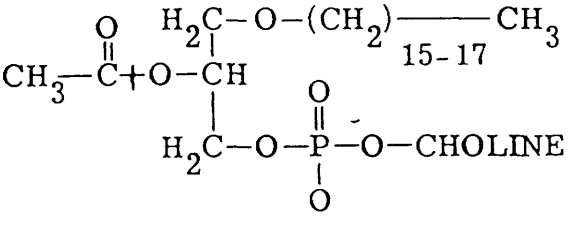


Figure 4. Structure of platelet activating factor 1-O-alkyl-sn2-acetyl glycerol phosphocholine (PAF) also known as PAF acether

4.2. PLATELET ACTIVATING FACTOR.

Platelet-activating factor (also known as PAF, PAF-acether or AGEPC) is synthesised by a number of different cell types including rabbit basophils, human and rabbit polymorphonuclear leukocytes and monocytes, rabbit & human platelets, human endothelial cells, mast cells, murine peritoneal macrophages and rat renomedullary cells **(Benveniste & Arnoux, 1983).** Recently the chemical structure of this molecule has been elucidated by two independent groups **(Demopoulos et al, 1979; Benveniste et. al., 1979).** PAF is an ether phospholipid of which 1-O-hexadecyl/octadecyl-2-acetyl sn glycerol-3-phosphorylcholine are the current standards (fig 4). The 1-O-alkyl, sn-2 acetyl and the phosphorylcholine moieties are essential for optimum biological activity.

4.2.1. BIOSYNTHESIS.

PAF is not stored within the cell but is synthesized during activation. The most likely source of the PAF molecule is a membrane alkyl acyl glycerophosphocholine (alkyl acyl GPC). The enzymes involved in the generation of PAF from human platelets have not been fully assessed. However **Snyder et al (1980)** have examined other cell systems which release PAF and concluded that a cyclic activation/inactivation pathway exists. The reactions of this pathway are shown in fig (5). Hydrolysis of the sn-2-acyl moiety of alkyl acyl GPC by a

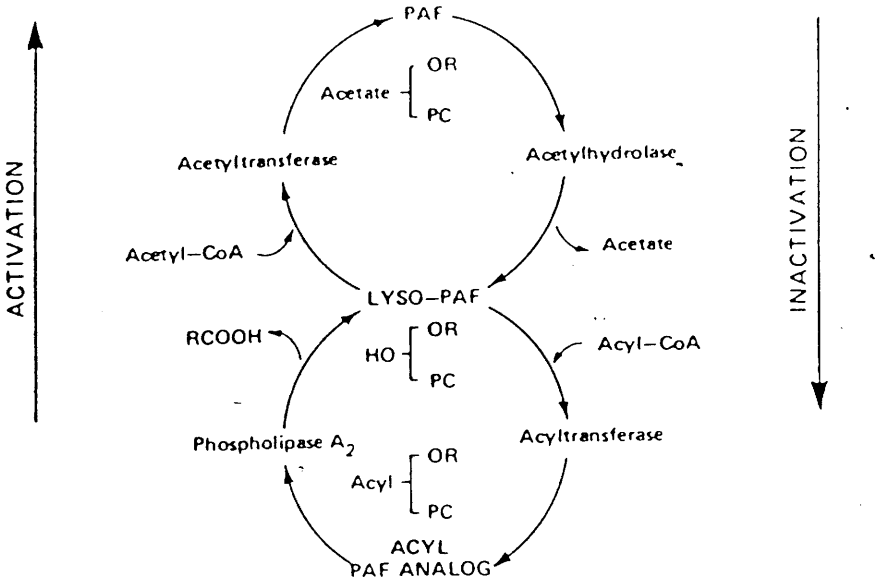


Figure 5. The reaction pathways involved in the activation and inactivation of Platelet Activatin Factor from its membrane precursor alkyl acyl glycerol phosphocholine. (Taken from Platelet Activating Factor (Benveniste, J. & Arnoux, B. eds.) INSERM symposium 23).

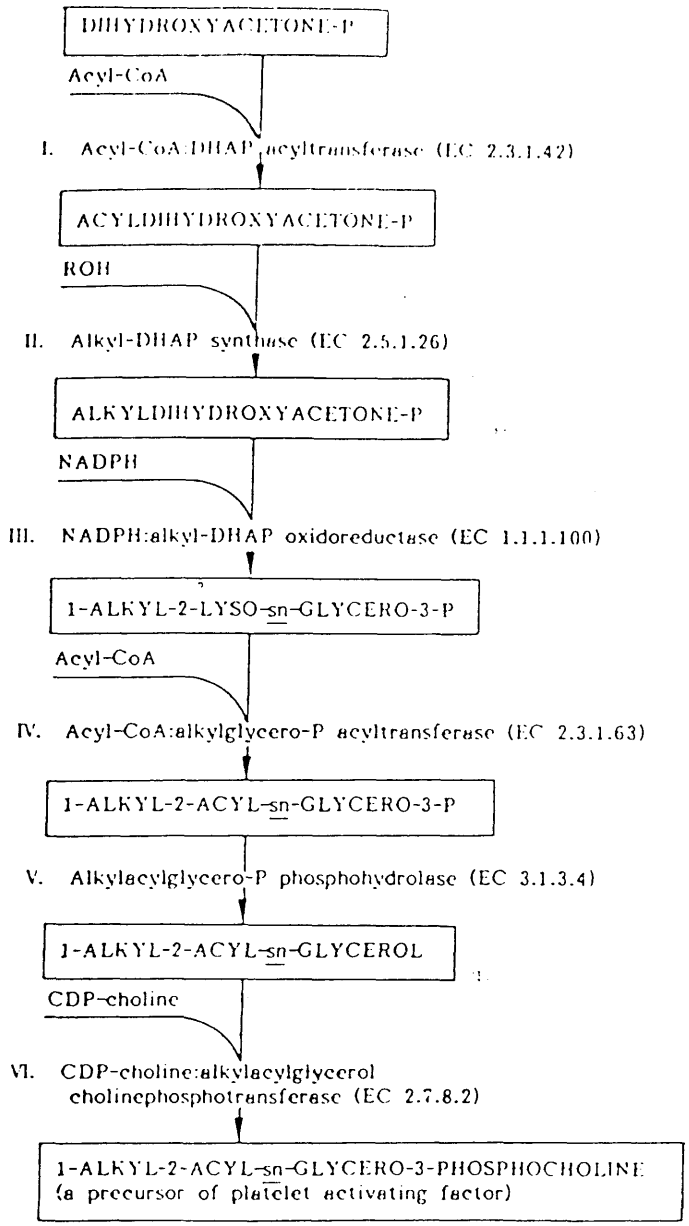


Figure 6. De-novo synthesis of alkyl acyl glycerol phosphocholine the membrane precursor of Platelet Activating Factor. (Taken from Platlet Activating Factor (Benvenise, J. & Arnoux, B. eds.) INSERM symposium 23).

phospholipase A_2 enzyme forms the biologically inactive Lyso-PAF. Subsequent acetylation of the sn-2 position by an acetyl transferase results in the formation of the active molecule. PAF is rapidly inactivated to Lyso-PAF by acetyl hydrolases which are apparently present both within the cell and in the plasma. Reacylation of Lyso-PAF regenerates the parent substrate alkyl acyl GPC.

Alkyl acyl GPC, the cellular source of PAF, may be synthesised de-novo via the pathway illustrated in fig (6). Activity of alkyl-DHAP synthetase, the crucial enzyme of this pathway; which catalyses the unique reaction forming the essential ether bond from acyl-DHAP and long chain fatty alcohols, has only been measured in rabbit platelets and alveolar macrophages **(Snyder et al, 1983).** However human platelets as well as many other cell types that release PAF contain a large proportion of their choline phospholipids as alkyl acyl GPC and therefore probably also contain this enzyme.

An alternative pathway for the synthesis of PAF involves a specific choline-phosphotransferase with alkyl acetyl sn-glycerol as the substrate. Much less information is known about this route.

Since activation of a phospholipase A_2 enzyme is also the mechanism underlying the mobilization of arachidonic acid , it is tempting to speculate that activation of PLA_2 releases both arachidonic acid and Lyso-PAF from the same membrane phospholipid. In PMN neutrophils **Swendsen et al (1983)** have shown that 1-O-alkyl-2-acyl sn-glycero-3-phosphorylcholine can act as a source of arachidonic acid. These cells form both PAF

and arachidonic acid from a single deacylation reaction. The evidence for such an event occuring in human platelets is less clear, **Smith (1984)** using labelled arachidonate has shown that 1-O-alkyl-2-acyl GPC is rich in arachidonate but no significant hydrolysis of the sn-2-acyl moiety occurs during stimulation. In contrast **Chignard (1984)** claims that a substantial amount (15%) of arachidonic acid originates from alkyl acyl GPC. The full extent of this molecule's biological activity remains to be established. The range of cell types which synthesis this lipid implies an involvement in a wide variety of biological events.

4.2.2. BACKGROUND

Initial observations, almost 40 years ago , noted that histamine was released from sensitized rabbit blood after immunological challenge. Rabbit platelets contain large quantities of histamine and later studies **(Barbaro & Zvaifler, 1966)** reported that the observed histamine release was mediated by a leukocyte-dependent mechanism. The involvement of a soluble factor was subsequently demonstrated by **Siraganian & Osler (1971)** and **Henson, (1970)**. The factor released from leukocytes caused platelet activation and was therefore named "Platelet Activating Factor" **(Benveniste, Henson & Cochrane, 1972)**. The specific leucocyte involved was identified as the basophil and the immune reaction was an IgE-dependent event. Much of the subsequent research concentrated on the role of PAF in immune/allergic and

inflammatory reactions and as a mediator of platelet activation.

In completely unrelated studies **Muirhead** revealed a cardiovascular action of PAF. The antihypertensive activity of renomedullary cells was originally noted by Muirhead in 1958. The active principle was found to be lipid in nature and was named "Antihypertensive Polar Renomedullary Lipid" (APRL) **(Muirhead et al, 1977).** A number of 1-O-alkyl phospholipids were identified in subsequent studies, of which PAF was the most conspicuous **(Muirhead et al, 1981).**

4.2.3. BIOLOGICAL EFFECTS.

Exogenous PAF induces thrombocytopenia, bronchoconstriction and hypotension in the guinea pig, monkey and rabbit **(Benveniste & Vargaftig, 1983).** The bronchopulmonary effects of PAF are platelet mediated in these species and can be separated from direct cardiovascular effects using platelet inhibitors. Rat platelets are refractory to PAF and hence only the platelet-independent effect of this agent, hypotension, is observed.

Although PAF can evoke inflammatory and allergic symptoms; is a potent vasodilator and platelet activator, its significance in pathophysiology is unclear. As an allergic mediator **Pinkard (1979)** has demonstrated that in vivo PAF is released into the plasma during the development of IgE-induced anaphylactic shock in rabbits, yet injection of PAF at the concentrations that produce vasodilation also produce thrombocytopenia,

bronchopulmonary effects and may initiate leukotriene biosynthesis and stimulate heart rate **(Vargaftig & Benveniste, 1983; Levi et al., 1984).**

In platelets **Chignard (1979)** has suggested that the generation of PAF may represent a pathway of platelet activation that is independent of released ADP or arachidonic acid, the so called "Third Pathway". Intrapulmonary platelet sequestration is associated with the condition Adult Respiratory Distress Syndrom (ARDS). It has been postulated that PAF, secreted from alveolar macrophages activates platelets in the pulmonary circulation which in turn release vasoconstrictors producing ARDS. Therefore PAF may have an important role in asthma **(Vargaftig & Benveniste, 1983; Levi et al., 1984).**

4.3. INOSITOL PHOSPHOLIPID METABOLISM.

Other lipid mediators of platelet activation are thought to include phosphatidic acid (PA), lysophosphatidic acid (LPA) and 1,2 diacylglycerol (DAG). Formation of these agents by stimulated platelets (and other cells) is thought to be intimately associated with inositol lipid metabolism which may also directly control platelet reactivity by regulating Ca++ "gating". Before discussing the effects of PA, LPA and DAG on platelets it is best to consider the current status (1984/5) of stimulated PI metabolism in cellular activation.

The inositol phospholipids constitute a minor component of the lipid membrane of most cell types comprising of about 10% of the total phospholipid content. The first indication that these phospholipids may participate in stimulus-response coupling came in 1953 when **Hokin & Hokin** observed an enhanced incorporation of ^{32}P into phosphatidylinositol (PI) of pancreatic cells following stimulation with acetylcholine. Since then this observation has been confirmed in numerous cell types and in response to a variety of agonists **(Berridge, 1981).** A common factor among all agonists that induce a "PI response" is that the cellular response is dependent upon an elevation of the intracellular free Ca++ concentration $[Ca^{++}]_i$.

4.3.1. PI RESPONSE OR PHOSPHOINOSITIDE CYCLE (Fig 7)

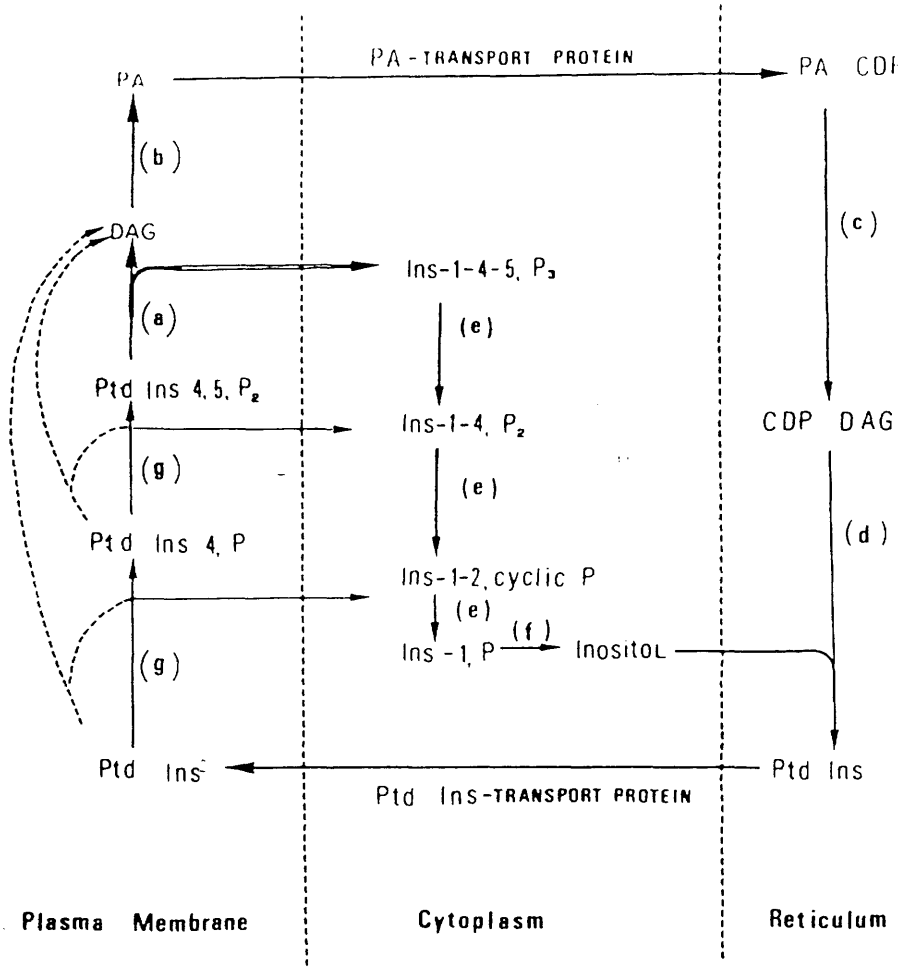


Figure 7. Inositol phospholipid hydrolysis.
The pathway description is given in text.

The PI response consists of an early and rapid hydrolysis of the inositol phospholipids followed later by their resynthesis. The inositol phospholipids are present in three forms phosphatidylinositol (Ptd Ins) and its phosphorylated derivatives phosphatidylinositol 4, bisphosphate (Ptd Ins 4P) and phosphatidylinositol 4,5, trisphosphate (Ptd Ins 4,5, P2). The initial event following stimulation appears to be the activation of a phospholipase C enzyme **(a)**. Since this enzyme is reportedly "Ca-independent", therefore active in the resting cell, the initial event may be the exposure of the appropriate inositol phospholipid substrate on the inner leaflet of the plasma membrane. Phospholipase C could act on all of the inositol phospholipids in each case resulting in the formation of diacylglycerol (DAG), which remains associated with the plasma membrane, and the hydrolytic inositol phosphates :- inositol 1-4-5 trisphosphate (Ins 1,4,5 P3) from Ptd Ins 4,5 P2; inositol 1-4 bisphosphate (Ins 1,4 P2) from Ptd Ins 4 P1 and inositol 1-2 cyclicphosphate (Ins 1-2 cyclic P) and inositol 1 phosphate (Ins 1 P) from Ptd Ins. However in liver cells the action of vasopressin results in a more rapid hydrolysis of Ptd Ins 4,5 P2 and Ptd Ins 4 P than Ptd Ins **(Kirk et. al., 1981). MacPhee et. al. (1983)** have shown similar findings in GH3 pituitary tumor cells in response to TRH. Therefore the predominant action of phospholipase C appears to be the hydrolysis of Ptd Ins 4 5 P resulting in DAG and Ins 1,4,5 P. This is probably followed by phosphorylation of Ptd Ins 4 P2 to Ptd Ins 4,5 P3 by specific kinases **(g)** in an attempt to maintain chemical equilibrium. DAG is rapidly

phosphorylated by diacylglycerol kinase **(b)** to phosphatidic acid (PA). PA and cytidine diphosphate (CDP) then form substrates for cytidine triphosphate-PA-cytidyl-transferase **(c)** forming CDP-diacylglycerol (CDP-DAG). Finally CDP-DAG-inositol-phosphatate transferase **(d)** reforms phosphatidyl-inositol. Inositol is rapidly reformed from the inositol phosphates by the action of a phosphodiesterase **(e)** and a phosphatase **(f)**.

The cellular location of these reactions are unclear although some evidence suggests that the hydrolysis occurs at the plasma membrane and resyntheses at the reticulum membrane (dense tubular system in platelets). In such a system the movement of PA and Ptd Ins across the aqueous phase (cytosol) would require a transport or carrier protein, the existence of such a protein has been reported **(Laffont et. al., 1981)**.

4.3.2. POTENTIAL PHYSIOLOGICAL ROLE OF PHOSPHOINOSITIDE HYDROLYSIS.

Virtually all agonists that induce a PI response evoke a calcium mediated cellular response. The correlation between the hydrolysis of PI and the generation of a calcium signal was first recognised by **Michell (1975)** who suggested that agonist receptor interaction stimulated an enhanced PI hydrolysis which subsequently "gates" calcium. The sequence of events proposed by Michell are the subject of intense debate. In challenging this view the opposition argue that the PI response is a consequence, rather than a cause, of an increased

availability of cytosolic free calcium. The credibility of Michell's proposal stems from the prediction that any cell which exhibits an early enhanced PI metabolism should always display a calcium mediated cellular response. This premise appears to hold true with one notable exception. In the adrenal medulla activation of nicotinic receptors can elevate intracellular calcium and evoke catecholamine secretion but do not cause a PI response whereas activation of muscarinic receptors elicit a PI response but do not elevate cytosolic calcium or cause release of catecholamines **(Ficher et. al., 1981).**

Perhaps the most important factor in determining whether or not the change in calcium precedes or follows the hydrolysis of PI is the calcium sensitivity of the latter. If PI metabolism is responsible for calcium "gating" then the enzymes involved in this response should be active at calcium concentrations associated with the resting cell or in other words they should be independent of changes in cytosolic free calcium. The majority of studies on this topic conclude that PI metabolism is essentially a calcium "independent" event **(Jones & Michell, 1978; Cockcroft & Gomperts, 1979; Kirk et. al., 1978; Fain & Berridge, 1979).** However some exceptions exist, notably the neutrophil **(Cockcroft et. al., 1980)** and pancreas **(Farese et. al., 1981)**, where the PI response is apparently calcium dependent.

Direct evidence for a causal relationship between PI hydrolysis and calcium "gating" comes from the work by **Berridge and Fain (1979)** who, using the blowfly salivary gland, successfully

depleted the membrane PI content and in parallel were able to show that calcium "gating" was severely diminished. Moreover when PI was restored calcium "gating" likewise recovered. Therefore, at least in this cell type, calcium "gating" would appear to be a consequence of PI metabolism .

4.3.3. RELATIONSHIP BETWEEN PHOSPHOINOSITIDE HYDROLYSIS AND CALCIUM GATING

HOW COULD THE PI RESPONSE "GATE" CALCIUM ?

Weiss & Putney (1981) have suggested that PI hydrolysis may be intimately associated with the opening of a receptor operated calcium channel thus allowing the influx of extracellular calcium. The immediate hydrolysis of Ptd Ins 4,5 P2 presents a second possibility. This inositol phospholipid is a potent calcium chelator, much of the membrane bound calcium is probably associated with this lipid **(Buckley & Hawthorne, 1972).**

In human platelets **Broekman (1984)** has demonstrated that the decrease in membrane bound calcium correlates with Ptd Ins 4, 5 P2 breakdown. Resynthesis of Ptd Ins 4, 5 P2, and chelation of cytosolic free calcium, should therefore display a negative correlation with cell-response which may indeed be the case **(Chap et. al., 1983).** Accordingly, its hydrolysis may represent a mechanism for releasing membrane bound calcium. However these phospholipids also bind Mg++ with only slightly less affinity than Ca++ and since the Mg++ : Ca++ concentration

is 10^3 : 1 it is more likely that the bulk of these lipids will bind Mg++.(**Fain 1982**)

Phosphatidic acid (PA), formed during the PI response , exhibits calcium ionophore activity in artificial systems (**Tyson et. al., 1976**). It has been suggested that the formation of PA may act as a natural ionophore allowing both influx of extracellular calcium and mobilization of intracellular calcium. PA accumulates rapidly in platelets (**Lapetina & Cuatrecasas, 1979**), in smooth muscle (**Salmon & Honeyman, 1980**) and in mammalian salivary gland (**Putney, 1981**). However in neutrophils (**Cockcroft et. al., 1980**) and pancreas (**Farese et. al., 1981**) PA formation lags behind the cellular response.

Finally **Streb (1983)** has demonstrated the ability of Ins 1,4,5 P3 to release calcium from intracellular Ca++-storage sites in the pancreatic acinar cell and more recently from purified platelet membrane fractions which were capable of Ca++-transport and thought to be DTS (**O'Rourke et. al., 1985; Authi & Crawford 1985**). The importance of Ins 1,4,5 P3 in regulating the availability of $[Ca^{++}]_i$ remains to be established conclusively. One important question that has yet to be answered is whether this metabolite can also evoke a calcium influx.

Agonist-induced inositol phospholipid hydrolysis may therefore directly control platelet reactivity by regulating the level of cytosolic free calcium. In addition several lipid metabolites of inositol phospholipid hydrolysis are considered to be

mediators of platelet reactivity The importance of these lipids in mediating platelet reactivity is discussed in the following sections.

4.3.4. 1, 2 DIACYLGLYCEROL .

1,2 diacylglycerol (DAG) is formed transiently during the course of inositol phospholipid hydrolysis and appears to represents a Ca++-independent second messenger. A ubiquitous kinase, protein kinase C, distinct from A and G kinases has been implicated in signal transduction in many cell types **(Nishizuka 1984).** PKC is a phospholipid- (principally phosphatidylserine) and Ca++- dependent enzyme which posesses multifunctional catalytic activity **(Nishizuka 1984).** However small amounts of DAG can dramatically increase the affinity of the enzyme for Ca++ conferring full activity in the absence of changes in the level of cytoplasmic Ca++ **(Kaibuchi et. al., 1981; Rink et. al., 1983).** Various reports show that tumor promoting phorbol esters (PMA) or a synthetic diacylglycerol, 1-oleoyl-2-acetyl-3-glycerol (OAG) can mimic the effect of endogenous DG by directly activating PKC **(Yamanishi et. al., 1983; Nishizuka 1984).** PMA can induce platelet aggregation and degranulation **(Zucker et al., 1974; White et al., 1974; Mufson et al., 1979).** Activation of PKC, in platelets, results in the phosphorylation of a 40 Kd protein **(Sano et al., 1983)** which has been reported by some workers as having a molecular weight of 47 Kd **(Imaoka et. al.,1983; Haslam & Davidson 1984).** Indeed initial studies demonstrated that OAG/PMA could induce secretion of dense body and lysosomal

constituents in the absence of changes in the level of cytosolic Ca++ and that these responses correlated closely with phosphorylation of the 40 Kd protein **(Yamanishi et al., 1983; Kaikawa et al., 1983).**

4.3.5. PHOSPHOLIPIDS

Two phospholipids phosphatidic acid (PA) and lysophosphatidic acid (LPA) are also reported to be platelet activators. PA accumulates rapidly in platelets **(Lapetina & Cuatrecasas 1979)**, and other cells following stimulation and has been shown to possess Ca++ ionophore activity in artificial membrane systems **(Tyson et. al., 197.)**. Its rapid formation in platelets could therefore elicit cellular responses by elevating the cytosolic level of Ca++. Indeed early reports demonstrated that exogenous PA could evoke platelet activation **(Gerrard et. al., 1978; Tokomura et. al., 1981; Gay et. al., 1968).** However in more recent experiments using very pure preparations of PA **Benton & Gerrard (1982)** were unable to induce platelet aggregation, even at high concentrations. Gerrard has suggested that contamination of commercial PA preparations by LPA may account for some erroneous observations **(Benton et. al., 1982).** Whether or not exogenous PA exhibits the same biological behaviour as endogenous PA remains to be determined.

LPA is also formed by activated platelets, exogenous LPA induces platelet activation **(Gerrard, 1977,1979; Tokomura 1981)** however, because the active concentration range of LPA

in platelets is 5 - 300 μM considerable doubt has been expressed over the ability of this lipid to act as an endogenous mediator as it seems unlikely that the cell could generate these concentrations during stimulation. **Lapetina et. al. (1981)** have estimated that the concentration of LPA formed during stimulation amounts to 5 - 10 % of the phosphatidic acid concentration. The amount of PA formed during supramaximal stimulation by thrombin is around 0.67 - 1.7 μM in a suspension of 3 x 10^8cells/ml **(Broekman, 1980).** Accordingly the concentration of LPA formed would be around 0.03 - 0.17 μM. Clearly this concentration would have little effect on platelet reactivity. However it has been reported **(Michell 1968)** that LPA generated during stimulation does not leave the cell. Thus, if LPA was retained within the cell, an intracellular concentration of 14 - 77 μM could be achieved **(Benton et al., 1982).** This being the case LPA could have a considerable effect on platelet reactivity. Like PA it remains to be proven whether the addition of exogenous LPA to cells mimics the effect of intracellular LPA. In addition Lapetina's estimate of the amount of LPA formed may be an underestimate since rapid reacylation of LPA may occur **(Lapetina et al., 1981).** Furthermore **Benton et. al. (1982)** have demonstrated that in the presence of solubilizing agents LPA has a significant effect on platelet reactivity at 0.25 μM.

4.3.6. ARACHIDONIC ACID.

Metabolites of inositol phospholipids are rich in arachidonate.

These include 1,2 diacylglycerol and PA. Arachidonic acid may be cleaved from 1,2,DG by either the direct action of a 1,2 diacylglycerol lipase (**Bell et al., 1979**) or the sequential action of a 1, 2 diacylglycerol lipase and a monoglycerol lipase (**Chau & Tai, 1983**) or from PA by the action of a specific PLA_2 (**Billah et al., 1981**). The liberation of arachidonic acid from inositol phospholipid metabolites has been discussed in section 4.1.1.

Many of the receptors associated with an elevation in the level of cytosolic calcium are also associated with an elevation of the intracellular level of cGMP (**Michell, 1975; Berridge, 1981**). Hence a further consequence of inositol phospholipid hydrolysis may be the activation of guanylate cyclase. The mechanism by which cGMP is elevated following receptor occupation is unknown. Although it has been suggested, because unsaturated fatty acid peroxides can activate platelet guanylate cyclase directly (**Hidaka & Asano 1977**), that the observed elevation of cGMP is mediated by an agonist-induced formation of arachidonic acid. Indeed in platelets it has been shown that blockade of cyclooxygenase can reduce collagen-induced formation of cGMP (**Haslam & McClenaghan, 1974**). However more recently it has been demonstrated that protein kinase C can directly activate Guanylate cyclase in vitro (**Zwiller et. al., 1985**).

4.4. AIMS OF STUDY

To characterise the effects and mechanisms of action of PUTATIVE LIPID MEDIATORS OF PLATELET ACTIVATION.

5. EXPERIMENTAL.

5.1. PREPARATION OF PLATELET RICH PLASMA.

Human blood was obtained by anti-cubital venepuncture from willing donors who denied taking any medication for at least 12 days. In certain specific instances blood was obtained from volunteers who had ingested aspirin (600mg) 24 hours previously. Whole blood (9 Vol.) was collected in plastic tubes, mixed with 0.13 M trisodium citrate (1 Vol.) and platelet rich plasma prepared by centrifugation (1000 g: 22°C: 5 min.). The platelet count was normally around 2 - 3 x 10^8/ml.

5.2. PLASMA FREE SUSPENSIONS OF PLATELETS.

Plasma free platelet suspensions (washed platelets) were prepared by gel exclusion chromatography using Sepharose 2B. PRP (30 % of gel bed volume) was passed through a perspex column containing sepharose 2B equilibrated with a modified Tyrodes buffer consisting of NaCl (129 mM), Na_3 Citrate (10 mM), Na HCO_3 (8.9 mM), Dextrose (0.56 mM), Trisma base (10 mM), KCL (2.8 mM), K H_2PO_4 (0.81 mM), Mg CL_2 (0.84 mM) and Ca CL_2 (2.4 mM); pH 7.4 at room temperature.

5.3. MEASUREMENT OF PLATELET AGGREGATION.

The technique used to monitor platelet aggregation was the method origi nally developed by **Born (1962)** and consists of monitoring the intensity of light passed through a stirred suspension of platelets (either PRP or washed platelets) before

and after the addition of a drug. The formation of platelet aggregates produces a decrease in optical density. Hence the increase in light transmission, monitored by a photoelectric transducer coupled to a chart recorder for permanent record, gives an index of platelet aggregation.

5.4. PLATELET SECRETION.

Platelet secretion (dense body release, alpha-granule release, lysosomal enzyme release and thromboxane B_2 (TxB_2 formation) was normally monitored in concert with platelet aggregation.

Using PRP which had been preincubated with [^{14}C]-5HT (1 µM final concentration: 50 nCi/ml) for 40 mim. at 37°C. Normal aggregation procedures were followed and at the appropriate time after agonist addition platelet reactions were terminated by the addition of 0.2 ml ice-cold EDTA (0.4 % (w/v)) in iso-osmotic saline containing imipramine (2 µM) to prevent 5HT reuptake. The contents were transfered to 1ml eppendorf tubes and the cells removed by centrifugation (8000 g; 4°C; 4 min.). Aliquotes of cell-free supernatant were used to measure secretion.

5.4.1. RELEASE OF DENSE BODY CONSTITUENTS.

Aliquots (100 ul) of cell-free supernatant were added to scintillation vials containing 10 ml triton:toluene:scintol 2 (12.3: 6.7: 1 v/v) for measurement of [^{14}C]-5HT release (R) (a marker for dense body release). Identical aliquots

containing platelet suspensions or cell-free supernatant from untreated platelets were also counted to obtain total (T) and background (B) radioactivity. Agonist-induced $[^{14}C]$-5HT release was calculated as a percentage of total platelet content according to the equation:-

$$\% \text{ RELEASE OF } [^{14}C]\text{5HT} = \frac{R - B}{T - B} \times 100\%$$

5.4.2. RELEASE OF ALPHA-GRANULE CONSTITUENTS.

Beta-thromboglobulin (BTG) (the marker used to monitor alpha-granule release) was measured using a BTG radioimmunoassay kit obtained from Amersham International, Amersham, UK. Briefly 50 µl of cell-free supernatant or BTG standards (10 - 225 ng/ml) were incubated with 200 ul of anti-BTG anti-serum and 200 µl of 125 I-BTG (40 nCi). The incubation tubes were sealed, vortex-mixed and allowed to equilibrate at room temperature for one hour. The antibody bound BTG was separated from the free BTG by precipitation with an ammonium sulphate solution (500 µl). After centrifugation (10,000 g: 22°C: 15 min.) and removal of the supernatant the radioactivity contained in the precipitate was counted using a New Egland Nuclear gamma counter. The ammount of BTG released was quantified by referring to a calibration curve comprising of BTG standards plotted against radioactivity bound.

5.4.3. RELEASE OF LYSOSOMAL ENZYMES.

B-N-acetylglucosaminidase (B-NAG), a one of the major platelet lysosomal enzymes, was monitored as a marker for release of lysosomal constituents. Aliquotes (100 µl) of cell-free supernatant or platelet pellet digested in 1 % triton X-100 (37°C: 30 min.) (enzyme source) were equilibrated at 37°C with 100 µl of 0.3 M sodium citrate buffer, pH 4.9, and the reactions initiated by adding 100 µl of 4-methyl-umbelliferyl-2-acetamido- 2-deoxy-B-D-glucopyranoside (3 mM) (substrate). After 60 min., the reactions were terminated by heating at 100°C for 2 min., and 700 µl of glass-distilled water was added to each sample. All samples were vortex-mixed, and 100 µl volumes of the diluted reaction mixture were added to 1.5 ml of 50 mM-glycine/NaOH buffer pH 10.4. The supernatant was clarified by centrifugation (14,000 g: 20°C: 1 min.) and the fluorescence of this solution measured in a Perkin-Elmer spectrophotofluorimeter with excitation and emission wavelengths of 370 nm and 450 nm respectively. Increases in supernatant fluorescence were expressed as a percentage of fluorescence in platelets of control, unstimulated platelets.

5.4.4. MEASUREMENT OF THROMBOXANE B_2 FORMATION.

Aliquotes (100 µl) of either cell-free supernatant or standard (10 pg - 1 ng TXB_2/ml) were incubated with 100µl of a 1/20.000 dilution of anti-TXB_2 anti-serum (the anti-serum was diluted in a potassium phosphate buffer (50 mM) containing

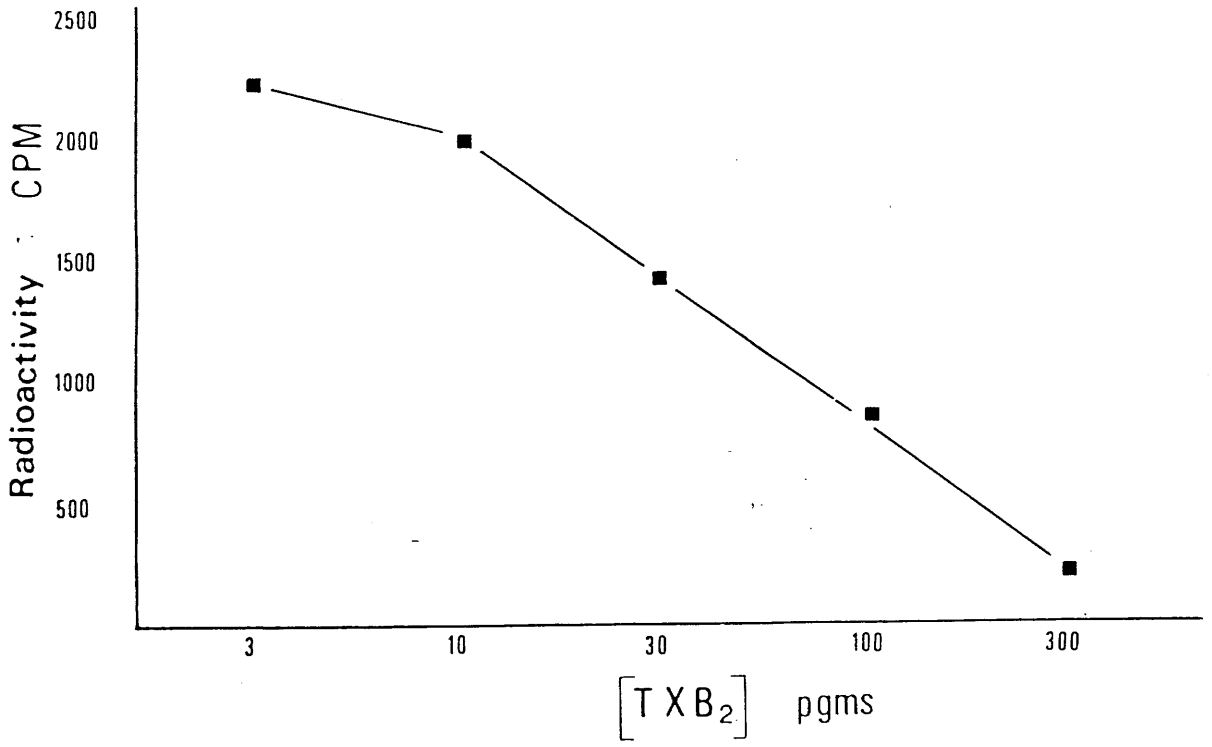


Figure 8. Thromboxane standard curve.

0.1 % (w/v) bovine gamma globulin, pH 7.3) and 200 µl of $[^3H]$-TXB_2. The incubation tubes were sealed, vortexed, and left to equilibrate for 18 hours at 4°C. Free TXB_2 was absorbed onto dextran-coated charcoal (100 µl) (0.5 % (w/v) charcoal: 0.5 % (w/v) dextran in a potassium phosphate buffer: pH 7.3) and removed by centrifugation (8,000 g: 4°C: 4 min.). Antibody bound $[^3H]$-TXB_2 was estimated by liquid scintillation counting. The amount of TXB_2 generated by PRP samples was quantified by reference to a calibration curve comprising known TXB_2 standards plotted against radioactivity bound. A typical curve is shown in fig. 8

5.5. MEASUREMENT OF CYTOSOLIC FREE CALCIUM ($[Ca^{++}]i$)

$[Ca^{++}]i$ was measured using the calcium indicator dye Quin2 **(Tsien, 1982).** PRP was incubated with 10 µM Quin2-acetoxy methyl ester (Quin2-AM) (37°C: 30 min.). In this form the dye readily permeates the plasma membrane, cytosolic esterases cleave the ester bond generating and trapping the active form (Quin2 free acid) of the dye in the cytoplasm. Loaded platelets were washed (to remove extracellular Quin2-AM) by gel filtration using sepharose 2B equilibrated with a modified Hepes/Tyrodes buffer consisting of Na CL (129 mM), Na_3 citrate (10.9 mM), Na HCO_3 (8.9 mM), dextrose (0.56 mM) Hepes (5 mM), KCL (2.8 mM), K H_2PO_4 (0.81 mM), Mg CL_2 (0.84 mM), Ca CL_2 (2.4 mM) and 0.35 % (w/v) bovine serum albumin pH 7.4 at 20°C.

The normal intracellular Quin2 concentration was 0.8 - 1 mM and nominally the free extracellular Ca++ was restored to 1 mM free

immediately before fluorescence measurement.

Platelet samples (1 ml) were contained in 1-cm square quartz cuvettes and maintained at 37°C. Fluorescence was recorded by an Amico-Bowman spectrophotofluorimeter coupled to a chart recorder, to secure a permanent record. Standard monochromator settings were:- excitation and emission 339 nm and 500 nm respectively.

The cytosolic calcium concentration $[Ca^{++}]_i$ was calculated form the following formula

$$Ca^{++}_i = Kd \frac{(F - F\ min.)}{(F\ max. - F)}$$

where F is the fluorescence recorded, F min. and F max. are the fluorescence recordings at very low and very high Ca++ respectively. Kd is the dissociation constant = 115 nM.

F max was determined by saturating Quin2 with Ca++. This was achieved by lysing the cells which exposed the entrapped dye to the high extracellular calcium concentration. Thereafter the addition of EGTA, to chelated Ca++, allowed the detemination of F min. During initial studies a number of techniques were employed to lyse the platelets. These included Triton X-100, ultra sonication and digitonin, of these digitonin (75 µM final concentration) provided the most consistent and reliable results and was therefore used throughout the experiments described.

5.6. MEASUREMENT OF PLATELET cAMP AND cGMP BY RADIOIMMUNOASSAY.

PRP or plasma free suspensions of platelets were treated with drug or vehicle for the appropriate time. Reactions were terminated and nucleotides extracted by the addition of ethanol (2 x vol.). Precipitated proteins were removed by centrifugation (15,000 g; 22°C; 30 mim.). The precipitate was washed with ethanol water (1 x vol.) (2 : 1 v/v) and the combined supernatants evaporated to dryness at 60°C under a constant stream of air. Efficiency of extraction was normally 95 %.

Radio-immunoassay methods were taken from **Steiner et. al. (1969), Brooker et. al. (1983)** and **Lappin & Whaley (1983).** Dried extracts or standards were dissolved in a sodium acetate buffer (50 mM: pH 6.2) and acetylated using a mixture of acetic anhydride:triethylamine (1 : 2 (v/v)) to improve assay sensitivity. 100 µl of either the unknown or standard (a) (0 - 500 pmoles cyclic AMP), (b) (0 - 500 fmoles cyclic GMP) were incubated with (a) 100 µl of a 1: 2000 dilution of rabbit anti- cyclic AMP anti-serum (the anti-serum was diluted in 0.1 (w/v) bovine serum albumin) or (b) 100 µl of anti-cyclic GMP anti-serum (1 : 300 dilution of anti-serum obtained from Amersham International, Amersham, UK. diluted in 0.1 % (w/v) bovine serum albumin) and [^{125}I]-succinyl cyclic AMP/GMP tyrosine methyl ester (2.66 nCi).

The incubation tubes were sealed vortexed and left to equilibrate for 18 hours at 4°C.

A 500 µl suspension of activated charcoal (0.25 % (w/v)) in a potassium phosphate buffer (100 mM:) bovine serum albumin: pH 6.2) was added to each incubation tube to absorb free

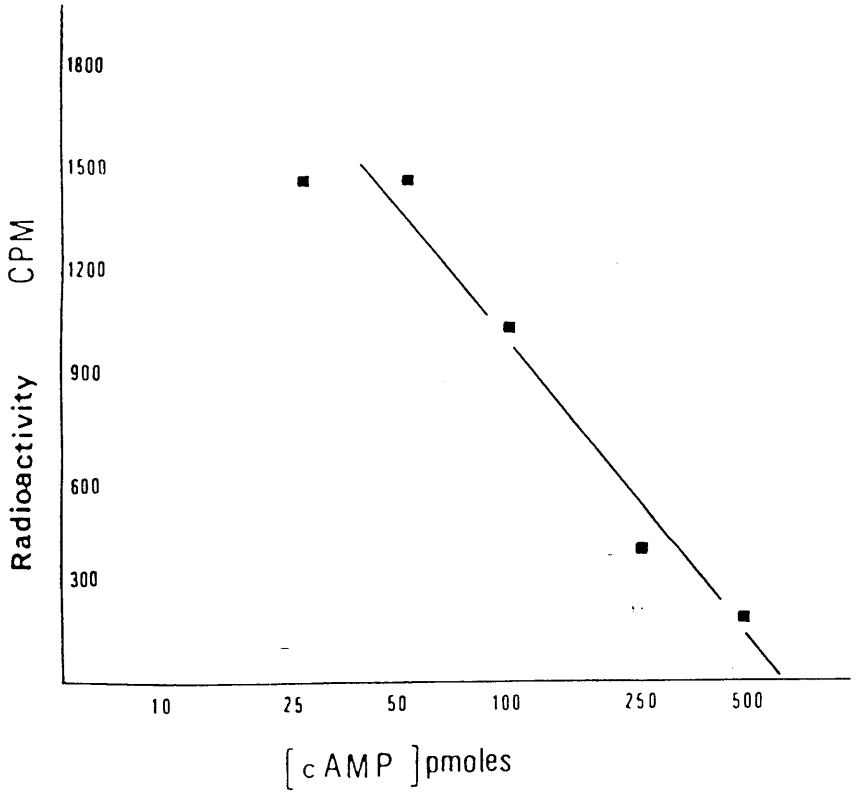


Figure 9a. Cyclic AMP standard curve.

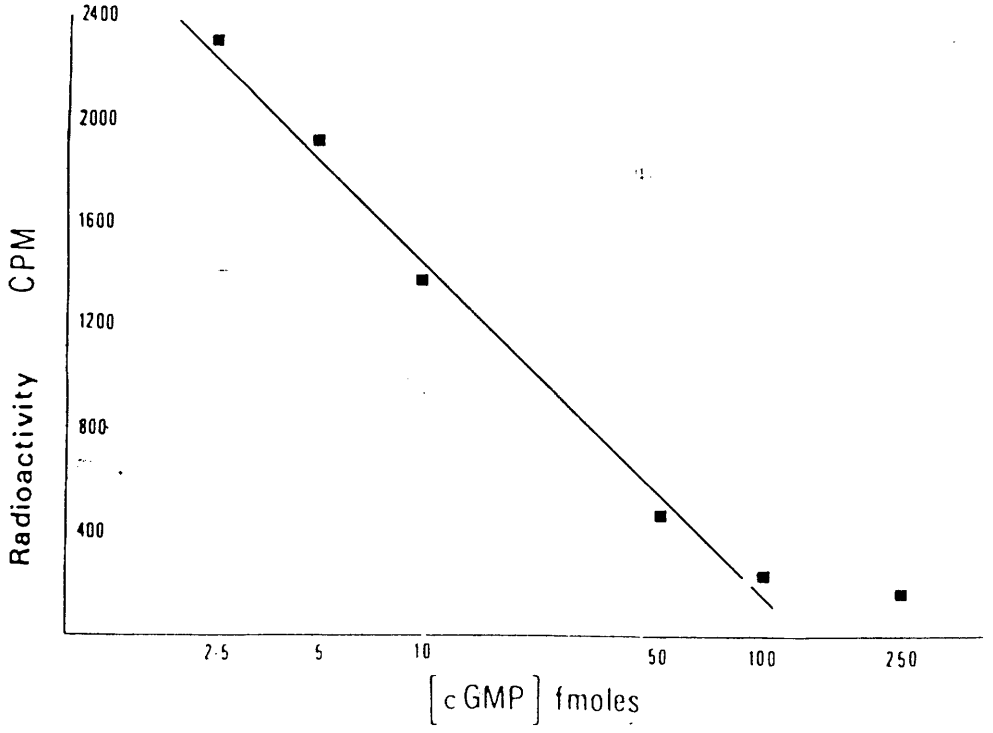


Figure 9b. Cyclic GMP standard curve.

nucleotide which was then removed by centrifugation (8,000 g: 2 min., 4°C) Aliquots (500 µl) of the supernatant, containing the antibody bound tracer, was counted in a New England Nuclear gamma counter. Cyclic nucleotides content was quantified by refering to calibration curves comprising of nucleotide standards plotted against radioactivity bound. Typical standard curves are depicted in fig. 9a and 9b

5.7. MEASUREMENT OF [^{3}H]-PAF BINDING TO RABBIT PLATELETS.

Rabbits were anaethetised by intravenous injection of Na-pentabarbitone and blood withdrawn, by polythene syringe (60 ml containing 1/10 vol. Na-citrate) from the abdominal aorta. 50 ml of whole blood was centrifu ed in 10 ml perspex tubes (50 g, 20°C, 5 min.) to remove contaminating red cells. This procedure was repeated until the PRP was essentially free of red cell contamination. Finally, in the presence of prostacyclin (1 µg/ml) platelets were pelleted by centrifugation (1000 g, 20°C, 12 min.), the supernatant decanted, and the cells resuspended in the modified tyrodes buffer described in section 5.5. ([Ca++]o = 1 mM)

Both labelled PAF (1-O-alkyl-1'-2' [^{3}H]--2-acetyl-sn-glycero-3-phosphorylcholine) ([^{3}H]-PAF) (New England Nuclear) and unlabelled PAF (1-O-hexadecyl-2-acetyl-sn-glycero-3-phosphorylcholine) were dissolved in methylene chloride:ethanol:water (20 : 10 : 1 v/v).

[^{3}H]-PAF (100 nCi : 200 pM - 500 nM) was added to 1 ml eppendorf tubes in the presence and absence of excess unlab.

PAF (10 μM). The ligand vehicle was reduced to dryness under a constant stream of oxygen free nitrogen and stored at - 20°C until required.

The incubation was initiated by the addition of 1 ml of a plasma free suspension of rabbit platelets and carried out at either 4°C or 20°C for three min. Incubations were terminated by rapid centrifugation (10,000: 4°C: 4 min.). The supernatant was carefully aspirated and the pellet digested with hyamine hydroxide at 60°C for 6 hours. The remaining solution was then neutralized with (N) HCL and 300 μl mixed with scintillation cocktail and counted for radioactivity.

Non-specific binding (counts recorded in the presence of excess ligand) were subtracted from total binding (counts recorded in the absence of excess ligand) to obtain specific binding.

5.8. MEASUREMENT OF PHOSPHATIDIC ACID FORMATION

A suspension of washed platelets was incubated, at 37 °C in the presence of carrier-free [^{32}P]-Phosphatidic acid (30 μCi/ml) for 90 min. Thereafter the platelets were pelleted by centrifugation (800 g for 10 min at 15 - 20 °C) in the persence of PGI_2 (0.3 μM) and resuspended in 1.5 times the labelling volume of fresh buffer after which the platelets were ready for use.

Radiolabelled platelets (0.4 ml) (containing approximately 0.5 - 1 mg of protein) were dispen ed into plastic tubes at 37°C. As a rough guide 10 ml of citrated whole blood yielded 3 aliquot s of labelled platelets. The maximum number of samples in any single experiment was 24 whilst within each experiment

triplicate determinations for each drug treatment were performed. Reactions were initiated by the additions of various concentrations of agonists (4 µl) in the presence of, or subsequent to, the addition of verapamil (4 - 10 µl) or the appropriate vehicle as a control. Reactions were terminated by transferring the entire platelet sample into a glass test-tube containing 2 ml of chloroform/methanol/10 M-HCl (25:50:4 v/v/v) at room temperature. Platelet lipids were extracted by partitioning of the aqueous and organic phases following the addition of 0.625 ml of chloroform and 0.625 ml of water according to the method of **Lloyd et. al. (1972).** After vortexing the tubes and centrifugation (1000 g for 15 min.) the lower (chloroform) phase was removed into a glass vial using a pasteur pipette, dried at 40°C under nitrogen and stored at -20°C until use. The lipids were redissolved in 0.15 ml of chloroform/methanol (9:1 v/v) and spotted on silica-gel t.l.c. plates (10 cm x 10 cm) for two-dimensional separation of phospholipids **(Yavin & Zutra, 1977).** This t.l.c. system employs a basic solvent in the first dimention chloroform/methanol/40% aqueous methylamine (13:6:1:5. v/v/v/v); neutralisation with HCl fumes; an acidic ether wash in the secnd dimention, diethyl-ether/glacial acetic acid (19:1, v/v) and an acidic solvent chloroform/acetone/methanol/glacial acetic acid/water (10:4:2:3:1, v/v/v/v/v), also in the second dimension. the major phospholipids separated by this method are shown in figure 10. In some cases 5 µg carrier phosphatidate was to facilitate identification of this lipid. Individual spots were detected by exposure of the plates to iodine vapour and/or

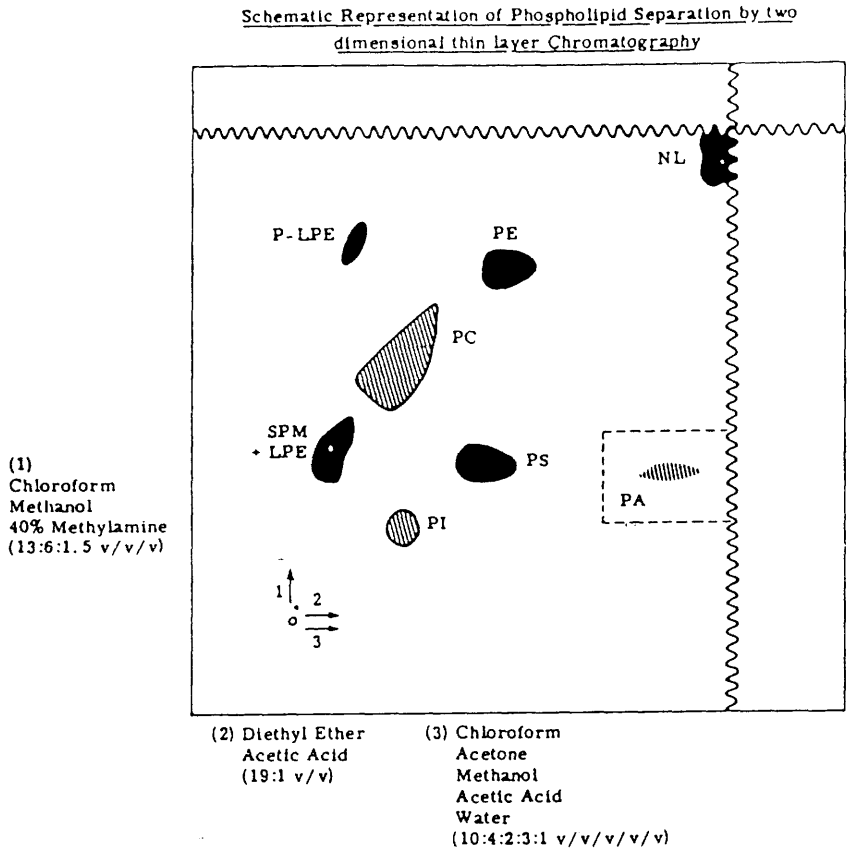


Figure 10. Schematic representation of phospholipid separation by two-dimensional thin layer chromatography. Phospholipids identified by iodine staining are indicated. The hatched spots indicate which phospholipids are significantly labelled with [^{32}P]Pi. O- origin; PI- phosphatidyl inositol; PC- phosphatidyl choline; PA- phosphatidic acid; PS- phosphatidyl serine; PE- phosphatidyl ethanolamine; P-LPE- plasmalogen lyso-phosphatidyl ethanolamine; SPM- sphyngomyelin; LPE- lyso-phosphatidyl ethanolamine; NL- neutral lipids.

autoradiography on X-ray film (15 - 20 hr.). A typical autoradiogram is shown in figure 10 phospholipids which incorporated radioactivity are indicated by the hatched lines.

6. THE EFFECTS OF PUTATIVE LIPID MEDIATORS ON PLATELET ACTIVATION AND THE ROLE OF ENDOGENOUS PLATELET MEDIATORS (ARACHIDONATE METABOLITES AND RELEASED ADP) IN THESE RESPONSES.

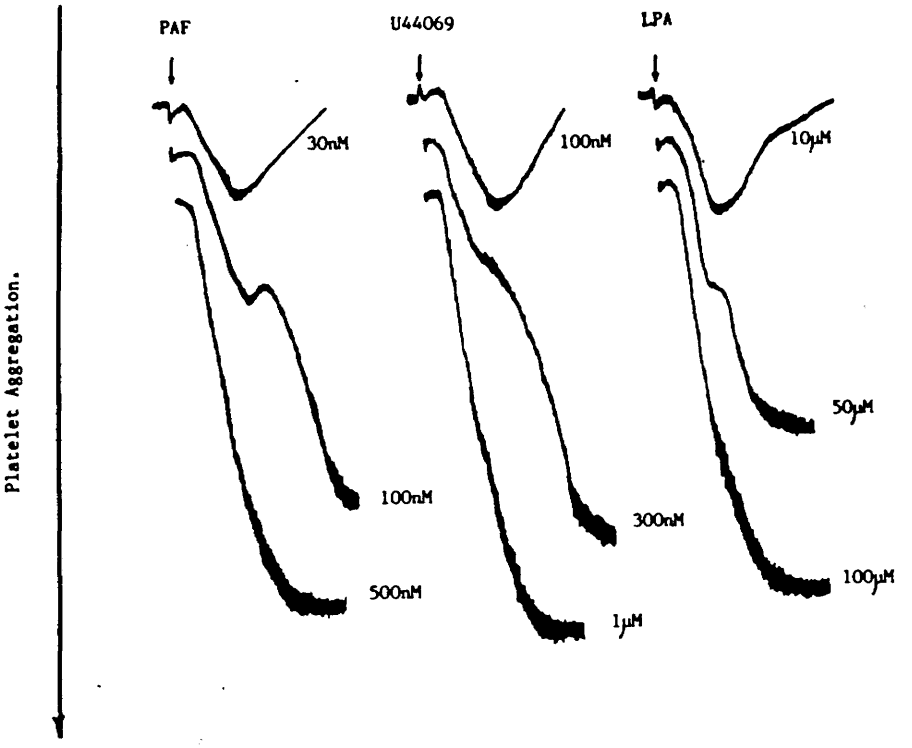


Figure 11. Platelet aggregation induced by PAF, U44069 and LPA. Agonists were added, at the concentrations indicated, to a stirred sample of PRP at 37°c to initiate aggregation.

Agonist	Aggregation	Degranulation Markers (% of Total) 5HT	βTG	βNAG	TXB_2 (ng/10^8 Cells)
PAF 0·1μM	R	0	0	0	0
PAF 0·5μM	I	25±8	83±6	12±4	1·8±0·25
PAF 5 μM	I	58±2	82±6	24±8	3·0±0·26
LPA 10 μM	R	0	0	0	0
LPA 50 μM	I	46±3	82±6	9±2	2·06±0·3
LPA 200μM	I	58±2	84±6	27±7	2.86±0·2
U44069 0·2μM	R	0	0	0	-
U44069 1 μM	I	37±10	70±8	0	-
U44069 5 μM	I	64±8	90±5	2	-

R = reversible.
I = irreversible.

Table 2. Platelet activation induced by PAF, LPA and U44069. Agonists were added at the concentrations indicated to stirred samples of PRP. After 2 minutes reactions were terminated by the addition of 1 vol. EGTA/imipramine as detailed in experimental. Thereafter cells were removed by centrifugation and aliquots of cell free supernatant assayed to determine the concentration of 5HT, TXB_2, BNAG and BTG.
The results are the mean values from 4 - 8 experiments usually conducted in duplicate.

6.1. EFFECTS OF PAF, LPA AND U44069 ON PLATELET ACTIVATION

PAF, LPA, PGG_2, PGH_2 and TXA_2 are putative endogenous mediators of platelet activation. Due to their instability and/or bioconversion PGG_2, PGH_2 and TXA_2 cannot be investigated directly consequently I utilized the stable analogue U44069 which mimics the effect of endoperoxides/TXA_2 on platelets to assess the effect of these icosanoids. In this section I characterised the effects of these putative lipid mediators on the various aspects of the platelet activation process and assessed the importance of endogenous AA metabolites and released ADP.

PAF, U44069 and LPA induce a concentration dependent activation of human platelets. Low concentrations of PAF (10 - 100 nM), U44069 (10 - 100 nM) and LPA (1 - 30 μM) induced , monophasic reversible platelet aggregation not associated with platelet secretion (primary aggregation). At higher concentrations all three agonists induced a monophasic or biphasic irreversible aggregation response (fig. 11) which was associated with the release of $[^{14}C]$-5HT (a marker for dense body secretion), BTG (a marker for alpha-granule secretion) (ie. secondary aggregation) and TXB_2 formation induced by PAF and LPA. U44069, which is structurally simillar to TXB_2, cross reacted with anti-TXB_2 antiserum and hence could not be measured. B-NAG (a marker for lysosomal secretion) release was also induced by PAF and LPA but not by U44069.

Table (2) summarises the effects of all three agonists on human platelet activation

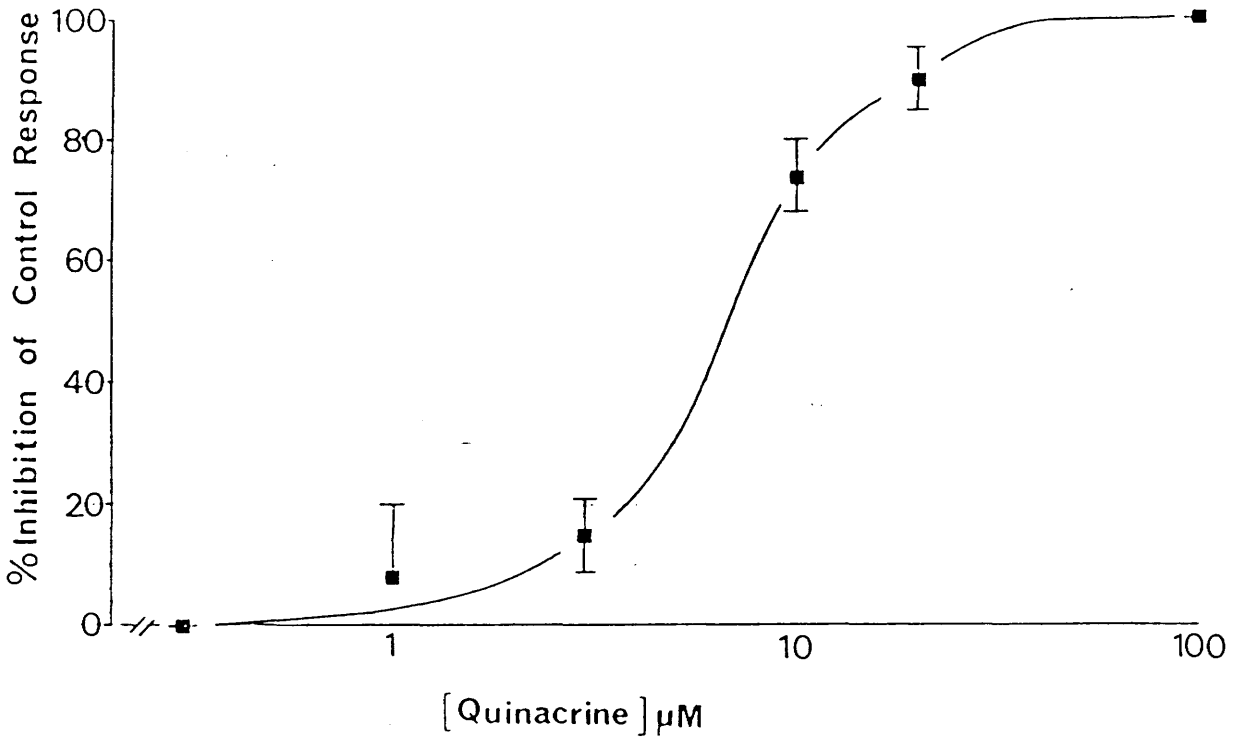


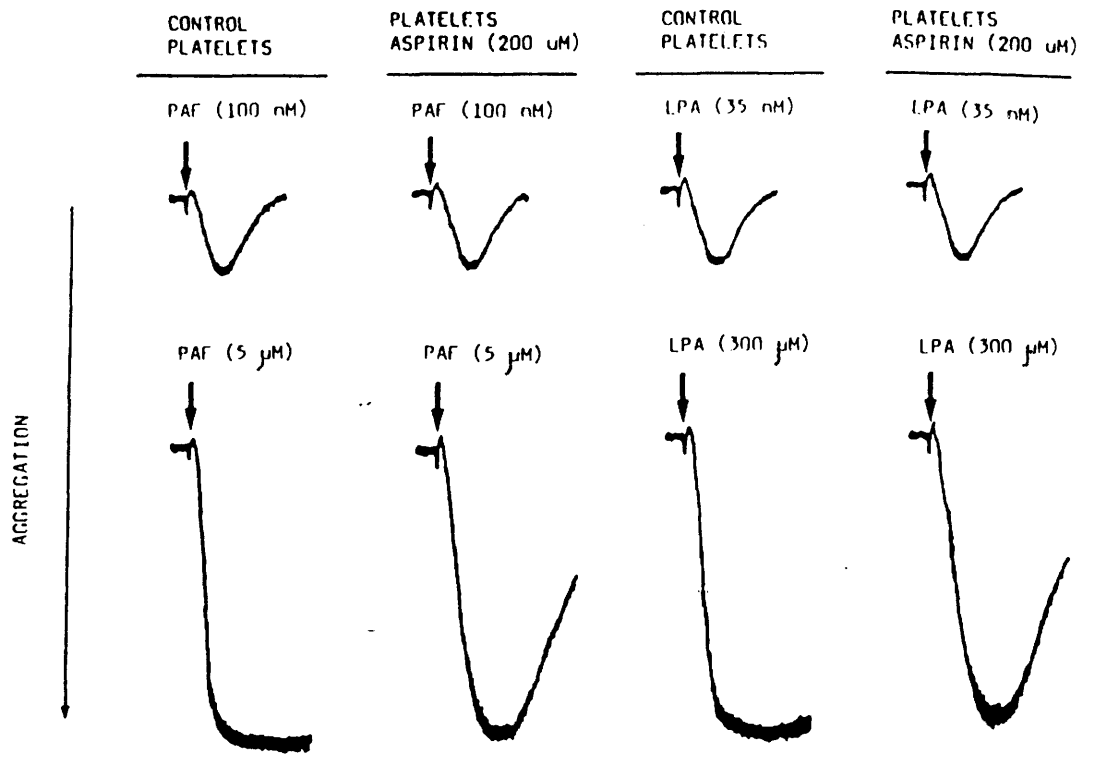
Figure 12. The effect of quinacrine on PAF-induced platelet aggregation Increasing concentrations of quinacrine ABSCISSA were preincubated with stirred samples of PRP for 2 minutes before the addition of PAF which induced sumaximal platelet aggregation. The ordinate shows the % inhibition of the control response. Results are the mean values ± S.E. from four experiments.

6.2. THE ROLE OF ENDOGENOUS ARACHIDONIC ACID METABOLITES AND ADP IN PAF- AND LPA-INDUCED PLATELET ACTIVATION.

In vitro platelet activation is normally initiated by exogenous agonists but propagated by endogenous platelet mediators, these include the cyclooxygenase metabolites of arachidonic acid (PGG_2, PGH_2 & TXA_2) and ADP released from platelet dense bodies. Agents which interfere with the various stages of arachidonate metabolism or which compete for the receptors for these agents were employed to assess the contribution of the endogenous mediators to platelet functional response - aggregation and dense body release (monitored by measuring [^{14}C]-5HT release) induced by PAF and LPA.

6.2.1. THE EFFECT OF A PLA_2 INHIBITOR.

Quinacrine has been reported to act as a selective inhibitor of PLA_2 **(Lapetina et al. 1981).** The effect of quinacrine on PAF-induced submaximal secondary aggregation is shown in figure (12). Quinacrine inhibited aggregation induced by PAF. The inhibition was concentration-dependent and at high concentrations the agonist response was abolished completely, implying that the effect was non-specific since inhibition of PLA_2 alone should only suppress secondary aggregation without influencing "primary aggregation". A similar effect OF quinacrine was observed when LPA was the stimulating agonist (ie. non-specific inhibition) (data not shown).



$[^{14}C]$ - 5HT RELEASE (% OF TOTAL)	64 ± 7	0	62 ± 5	0
TXB_2 ($ng/10^8$)	2.7 ± 0.05	0.18 ± 0.03	2.5 ± 0.07	0.18 ± 0.03

Figure 13. The effect of aspirin on PAF- and LPA-induced platelet activation. Supramaximum concentrations of agonists were added to stirred samples of PRP obtained from donors who had ingested aspirin (3 mg) at least 3 hours before donating. Reactions were terminated after 2 minutes of agonist stimulation by the addition of 1 vol. of EGTA/imipramine. Cells were removed by centrifugation and aliquotes of cell free supernatant assayed for 5HT and TXB_2 content. The results are the mean values ± S.E. from 4 - 8 experiments.

6.1.2. THE EFFECT OF CYCLOOXYGENASE INHIBITION.

In the presence of aspirin (200 μM) TXB_2 formation, monitored as an index of cyclooxygenase activity was abolished; remaining at basal levels (0.18 ± 0.03 ng/10^8 cells; S.E. from 4 - 8 experiments conducted in duplicate). Under these conditions primary aggregation induced by PAF or LPA was unaffected. The extent of aggregation induced by supramaximal concentrations of PAF (5 μM) or LPA (300 μM) was unaffected by aspirin although the form had changed from an irreversible to a reversible response fig (13). 5HT release was completely abolished in the presence of aspirin.

6.2.3. THE EFFECTS OF THROMBOXANE SYNTHETASE INHIBITION.

The effect of aspirin clearly indicates that the presence of cyclooxygenase metabolites of arachidonic acid (PGG_2, PGH_2 & TXA_2) are essential to achieve dense body secretion and irreversible aggregation induced by PAF and LPA. Under normal conditions the prostaglandin endoperoxides are rapidly converted to TXA_2 by thromboxane synthetase. The importance of TXA_2 alone in mediating dense body secretion and irreversible aggregation, induced by PAF and LPA, may be assessed by employing the thromboxane synthetase inhibitor dazoxiben **(Randall et al., 1981)**. TXB_2 formation was monitored as an index of TX-synthetase activity. When compared to controls, in which PAF (5 μM) and LPA (300 μM) induced the formation of 2.7 ± 0.05 and 2.5 ± 0.07 ng $TXB_2/10^8$ cells respectively, TXB_2 levels in platelets treated with

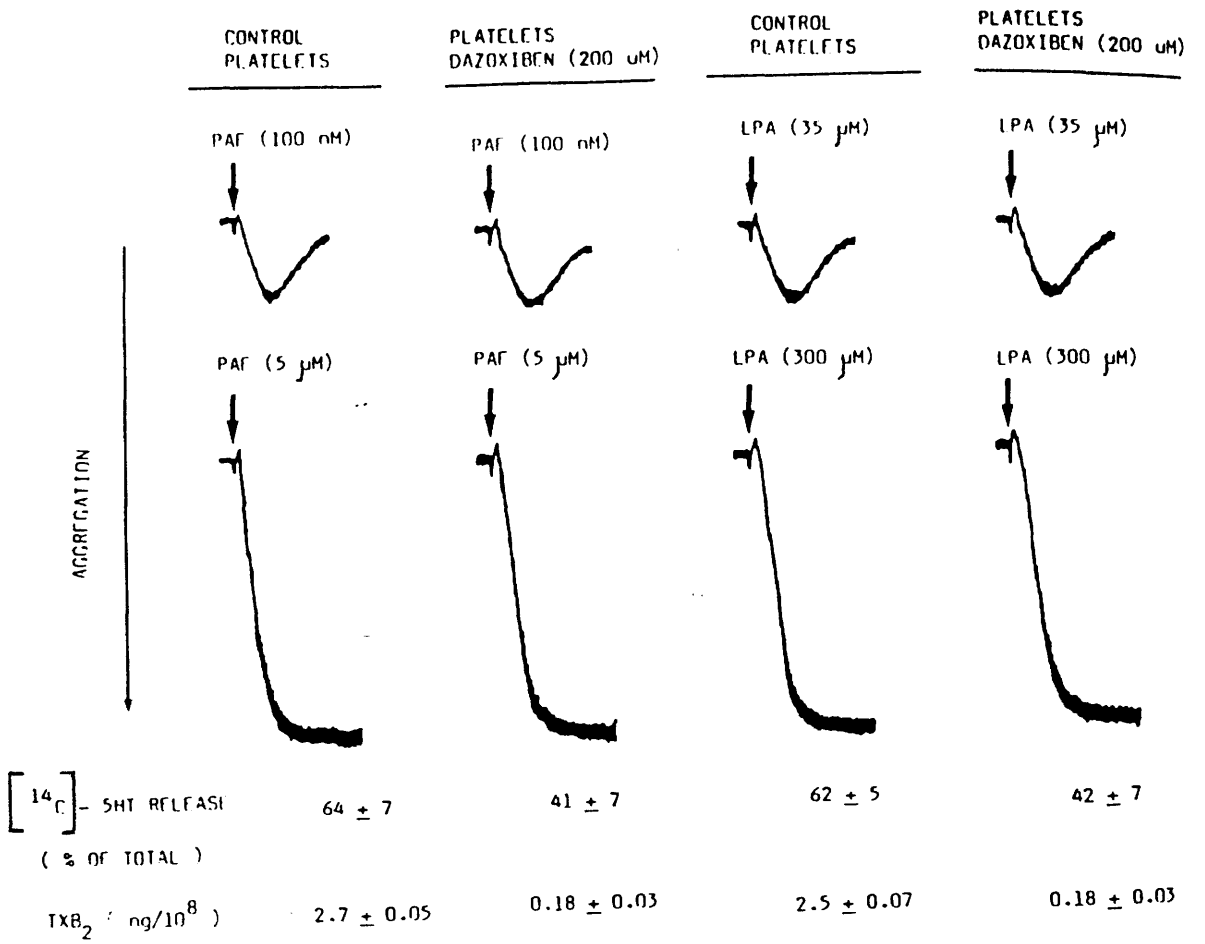


Figure 14. The effect of dazoxiben on PAF- and LPA-induced platelet activation. Dazoxiben (100 uM) was added to stirred samples of PRP. 2 minutes latter supramaximum concentrations of agonists were added. After 2 minutes of agonist stimulation the reactions were terminated as described for the previous figure. Results are the mean values ± S.E. from 4 - 8 experiments conducted in duplicate.

the TX-synthetase inhibitor dazoxiben remained at basal values (0.18 ± 0.03 ng/10^8 cells (fig. 14). Thromboxane synthetase inhibition had no effect on either "primary" or "secondary" aggregation induced by PAF or LPA. [^{14}C]-5HT release was reduced from 60 % (controls) to 40 % in the presence of dazoxiben (means ± S.E. from 4 - 8 experiments conducted in duplicate).

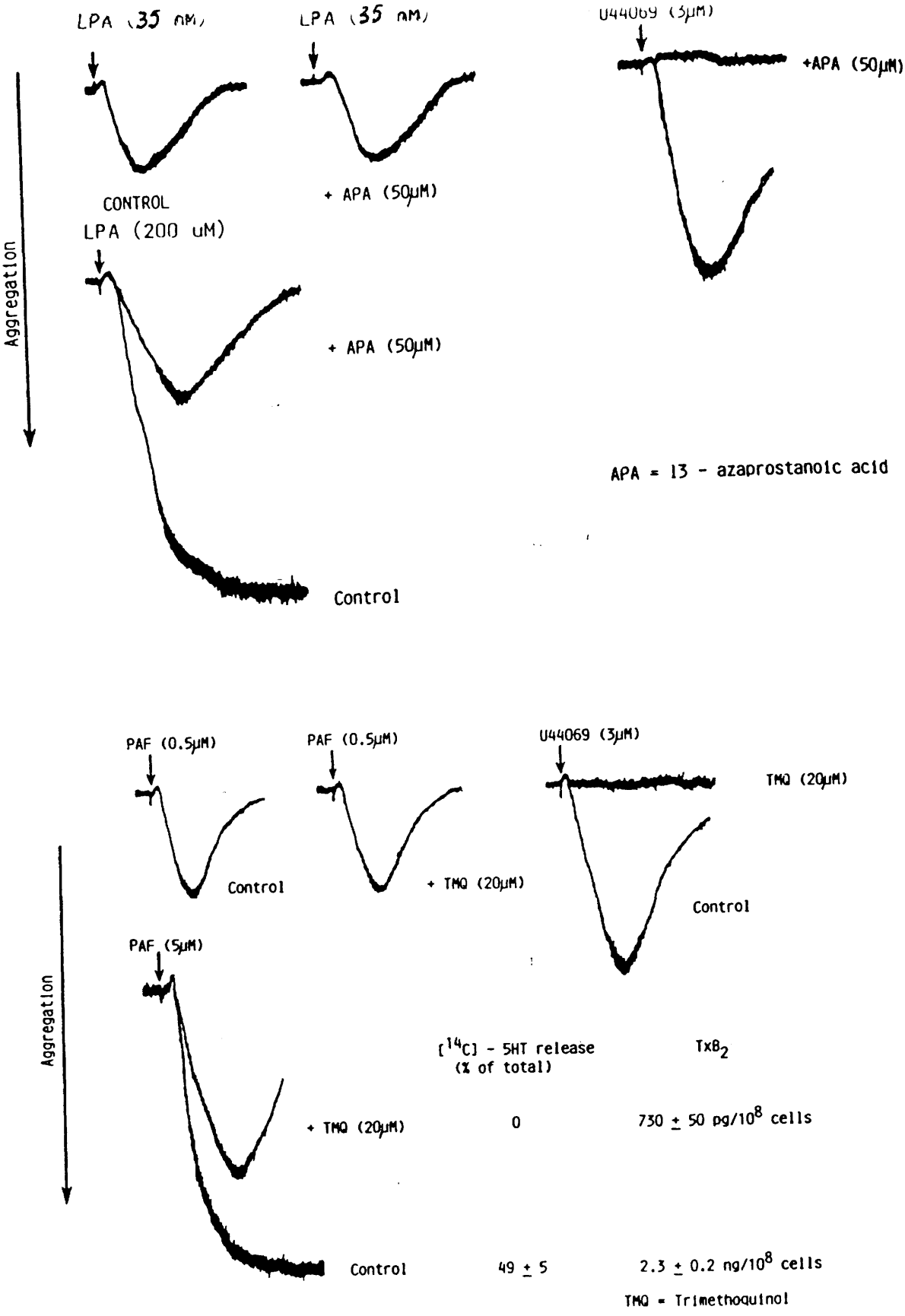


Figure 15. The effect of 13 azaprostanoic acid (13-APA) and trimethoquinol (TMQ) on PAF- and LPA-induced platelet activation. 13-APA/TMQ were preincubated for 2 minutes in stirred samples of PRP. Platelets were then stimulated with supramaximum concentrations of PAF or LPA and the reactions terminated 2 minutes later by the addition of EGTA/imipramine . Thereafter the 5HT and TXB_2 content in aliquots cell free supernatant was assayed. The results are the mean values ± S.E. from 3 - 4 experiments conducted in duplicate.

6.2.4. THE EFFECT OF PROSTAGLANDIN ENDOPEROXIDE/THROMBOXANE A_2 RECEPTOR ANTAGONISM.

An intact cyclooxygenase is apparently essential to achieve $[^{14}C]$-5HT release and irreversible aggregation induced by PAF and LPA. Yet dazoxiben, which abolishes the formation of the principle metabolite of the cyclooxygenase pathway (TXA_2) has no effect on aggregation and only inhibited $[^{14}C]$-5HT release by 30 % suggesting that the endoperoxides are capable of mediating secondary aggregation and dense body release. Hence agents which inhibit the binding of the endoperoxides and thromboxane to their common receptor in platelets would be expected to mimic the response recorded in the presence of aspirin.

The effect of two endoperoxide/TXA_2 receptor antagonists trimetoquinol (TMQ) **(MacIntyre & Wills 1978)** and 13 azaprostanoic acid **(Le Breton et al., 1981)** (13-APA) on PAF- and LPA-induced secondary aggregation are illustrated in fig (15). At concentrations which abolish aggregation induced by the TXA_2 mimetic U44069, 13-APA (50μM) reversed the secondary response but had no effect on primary aggregation induced by either PAF or LPA. Figure 15a shows a typical aggregation trace induced by LPA in the presence and absence of 13-APA.

TMQ displayed the same inhibitory pattern as 13-APA. In the presence of TMQ TXB_2 formation induced by PAF (5 μM) or LPA (200 μM) was inhibited by 70 % from 2.3 $\pm$ 0.2 (untreated control cells + PAF) and 2.2 $\pm$ 0.3 (untreated cells + LPA)

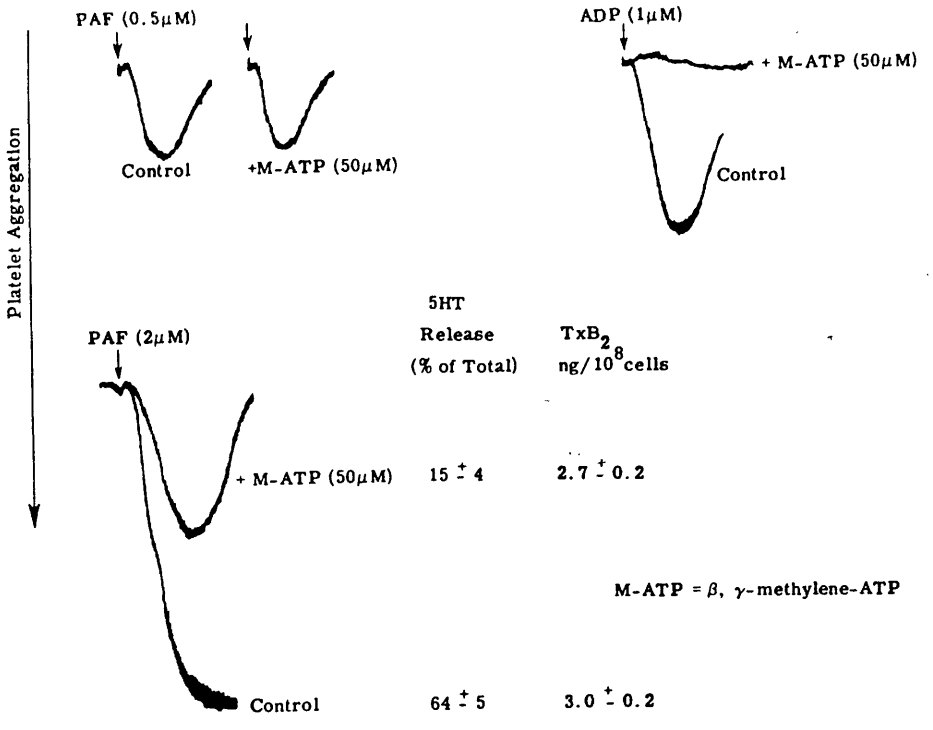


Figure 16. The effect of Beta-Gamma-Methylene-ATP (B-γ- Me-ATP) on PAF-induced platelet activation. B-γ- Me-ATP was preincubated in stirred samples of PRP for 2 minutes. Supramaximum concentrations of PAF were added to initiate platelet activation and after 2 minutes the reactions were terminated by the addition of EGTA/imipramine. Aliquots of cell free supernatant were then assayed for 5HT and TXB_2 content. The results are the mean values from 3 - 4 experiments conducted in duplicate.

ng/10^8 cells to 730 ± 50 pg/10^8 cells in the presence of TMQ (20 μM) (means ± S.E. from 3 - 4 experiments conducted in duplicate). PAF- and LPA-induced [^{14}C]-5HT release was abolished by TMQ. Figure 15b shows the effect of TMQ on PAF-induced platelet aggregation, 5HT release and TXB_2 formation.

6.2.5 THE EFFECT OF BETA-GAMMA-METHYLENE-ATP ON PLATELET AGGREGATION AND DENSE BODY RELEASE INDUCED BY PAF AND LPA.

Beta-gamma-methylene-ATP (B-γ-Me-ATP), an ADP receptor antagonist **(Cusack et al., 1985)**, was employed to examine the contribution of released ADP to secondary aggregation induced by PAF and LPA. B-γ-Me-ATP, at concentrations which abolished aggregation induced by 1 μM ADP, reversed the secondary aggregation response induced by 2 μM PAF or 200 μM LPA but had no effect on primary aggregation induced by either PAF or LPA. PAF (5 μM) and LPA (200 μM) induced the formation of 3.0 ± 0.2 and 2.3 ± 0.2 ng TXB_2 /10^8 respectively. In the presence of B-γ- Me-ATP no significant reduction in TXB_2 was observed (2.7 ± 0.1 and 2.2 ± 0.1 ng TXB_2 /10^8 for PAF and LPA respectively). However 5HT release induced by PAF and LPA was reduced from 60 ± 4 % to 15 ± 4 % and 52 ± 7 % to 13 +5 % respectively; mean values ± S.E. from 4 experiments conducted in duplicate. Figure 16 shows the effect of B-γ-Me-ATP on PAF-induced aggregation, 5HT release and TXB_2 formation.

6.3. CONCLUSION.

It is well established that human platelets can generate TXA_2 and recently it has been shown that PAF **(Chignard et al., 1980)** and LPA **(Schumacker et al., 1979)** are also produced during physiological stimulation.

PAF, PLA and U44069 when added to human PRP induce primary aggregation at low concentrations and induce secondary aggregation at higher concentrations. Because they induce the selective release of constituents from the platelet dense bodies, alpha granules and lysosomes PAF and LPA may classified as powerful platelet agonists **(Holmsen, 1972)**. In contrast U44069 does not induce lysosomal enzyme secretion and thus may be classified as a weak platelet agonist.

The release of dense body constituents and irreversible aggregation by PAF or LPA requires the formation of TXA_2 though in the absence of TXA_2 the endoperoxides are only slightly less effective in mediating these responses. Released ADP is also necessary, in addition to TXA_2 or the endoperoxides, to achieve irreversible aggregation. Released ADP apparently also acts as a positive feedback mechanism for release of dense body constituents.

7. THE ROLE OF CYTOSOLIC FREE CALCIUM IN PLATELET ACTIVATION.

Electrophysiological studies have established that the concentration of cytosolic free calcium is an important intermediary regulating stimulus -secretion coupling **(Silinsky, 1982)** and stimulus-contraction coupling **(Breeman et al., 1973).** Because of their size conventional electrophysiological techniques cannot be applied to the study of platelet physiology. Until now a central role for calcium in modulating stimulus-response coupling in these cells has been largely circumstantial, being inferred from studies using the divalent ionophore A23187 **(Feinstein, 1980; Gerrard, 1981)** (see section 3) and by analogy with other cell systems; platelet functional response also includes active contraction and secretion of granule constituents. Only recently with the development of the Quin2 technique, has direct evidence been available. In an attempt to elucidate the role of cytoplasmic calcium in stimulus response coupling in platelets first of all I examined, using Quin2, the effect of various agonists, including PAF, LPA and U44069, on $[Ca^{++}]_i$ in human platelets. Electrophysiological studies also have established that agonist-induced changes in $[Ca^{++}]_i$ may arise through mobilization of Ca++ from intracellular storage sites and/or influx of extracellular Ca++ via channels, pores or carriers in the plasma membrane **(Bolton, 1979; Breeman et al., 1973)** That similar mechanisms (ie. influx or mobilization) operate in/on platelets has again been inferred from studies using the calcium ionophore A23187 **(Massini & Luscher, 1974)** (see section 3). By monitoring any change in $[Ca^{++}]_i$ in the presence and absence of EGTA, to chelate extracellular Ca++, I next

sought to establish the possible source of $[Ca^{++}]_i$. The assumption being that changes in $[Ca^{++}]_i$ monitored in the absence of external Ca++ cannot be due to influx and therefore must be attributed to mobilization of Ca++ from intracellular storage sites to the cytosol.

Various studies have shown that stimulated platelets take up $^{45}Ca^{++}$ implying that an influx of extracellular Ca++ occurs upon stimulation **(Owen et al, 1980; Massini & Luscher, 1974)**. Two types of calcium channel (or pore), which allow an influx of extracellular Ca++ down its electrochemical gradient have been identified. One which opens as the membrane is depolarized and the other which may admit Ca++ in the absence of changes in the membrane potential. The latter type is thought to be closely associated with agonist receptors and therefore has been called receptor operated channels (ROC's), while the former is referred to as a voltage operated channel (VOC). **(Bolton, 1979)**.

The types of Ca++-channel in the human platelet membrane have not been identified. **MacIntyre and Rink (1982)**, using the fluorescent probe 3,3-dipropylthia-dicarbocyanine (DI S-C3-5), reported that platelets stimulated by a number of agonists do not alter the membrane potential, suggesting that if an influx of Ca++ occurs it does so through ROC's. This report, however conflicts with that of **Greenberg-Sepersky and Simmons (1984)** who, using the same technique, reported a depolarization of the membrane (5 - 10 mV) following stimulation by thrombin and ADP.

Since VOC's are sensitive to changes in the membrane potential

it should be possible to open these channels selectively by depolarizing the trans-membrane potential. Platelets maintain large transmembrane gradients of potassium (K+) and sodium (Na+) **(Feinberg et al, 1977; Moake et al, 1970).** Because platelets maintain a low Na+ and high K+ permeability, at rest, the latter ion is the major contributor to the membrane potential; indeed the potassium equilibrium potential (Ek) = the membrane potential (Vm) = 60 mV **(MacIntyre & Rink, 1982)** The membrane can therefore be readily depolarized by suspending the cells in a medium containing a high concentration of K+.

Hence in the fourth study of this section I used Quin2 loaded platelets suspended in a medium containing high extracellular K+. In so doing, an elevation in $[Ca^{++}]_i$ evoked by membrane depolarization would establish the presence of VOC's. An alternative strategy makes use of the recently developed Ca++-agonist BAY K8644, a dihydropyridine (DHP) analogue which selectively opens or maintains open VOCs **(Yamamoto et al, 1984).** In Quin2 loaded platelets this agent should also elevate $[Ca^{++}]_i$ if VOCs are present. BAY K8644 has been shown to evoke a secretory response from electrically excitable cells such as adrenomedulla **(Montiel et al, 1984)** and GH3 pituitary cells **(Enyeart & Hinkle, 1984). Tsien (Hess et al, 1984)** has postulated that VOC's exist in three modes: 1) inactive, 2) short opening and prolonged closure and 3) prolonged opening and short closure. DHP agonists, which compete with DHP antagonists for a common binding site on the putative VOC **(Janis et al, 1984),** alter the gating kinetics favouring

mode 3 whereas the antagonist promotes modes 1 and 2. Moreover the effect of DHP agonists or antagonists is enhanced by membrane depolarization (so-called use-dependence) **(Hess et al, 1984).** Consequently incubating BAY K8644 with agonists which reputedly depolarize the membrane (thrombin and ADP) should potentiate any change in $[Ca^{++}]_i$ recorded in the absence of this agent.

It is evident from section 6 that release of dense body constituents induced by PAF and LPA requires the formation of the endoperoxides or thromboxane A_2. The mechanism by which these icosanoids induce the release of constituents of storage organelles is unknown. **Rink et al. (1982)** have shown that the various platelet functional responses can be triggered at different $[Ca^{++}]_i$ (see section 3.2.) suggesting that each functional response is triggered at a particular $[Ca^{++}]_i$ threshold. Therefore one possible mechanism by which these icosanoids might mediate PAF/LPA-induced dense body release would be to elevate $[Ca^{++}]_i$ to the threshold necessary to evoke this response.

In contrast to the cyclooxygenase metabolites it has been suggested that lipoxygenase metabolites of arachidonic acid might function as feedback inhibitors effectively limiting the extent of the cellular response (see section 4.1.5.). Such a mechanism could involve reducing the availability of the second messenger (ie. $[Ca^{++}]_i$). Inhibition of cyclooxygenase would be expected to reduce the agonist-induced elevation by both removing the effect of PGG_2,PGH_2 & TXA_2 (ie that part of the agonist-induced elevation evoked by

PGG_2,PGH_2 & TXA_2 receptor interaction) and by increasing the availability of arachidonic acid for metabolism by the lipoxygenase enzyme. Selective inhibitors of lipoxygenase are not available at present. However, agents which inhibit both lipoxygenase and cyclooxygenase are available, and in the present of such agents changes in $[Ca^{++}]_i$ would be expected to be due entirely to the initiating agonist. Hence in the fifth study of this section I examined the effect of cyclooxygenase inhibition, cyclooxygenase/lipoxygenase inhibition and also the effect of 12-HETE, one of the lipoxygenase metabolites which may act as a negative feedback inhibitor, on PAF-induced changes in $[Ca^{++}]_i$.

Finally if the cytosolic free calcium concentration regulates or mediates the platelet functional response then the concentration response curve for $[Ca^{++}]_i$ should correlate with the concentration response curve for some functional response. To investigate this I monitored $[^{14}C]$-5HT release (a marker for dense body secretion) in parallel with changes in $[Ca^{++}]_i$ induced by PAF, thrombin and ADP.

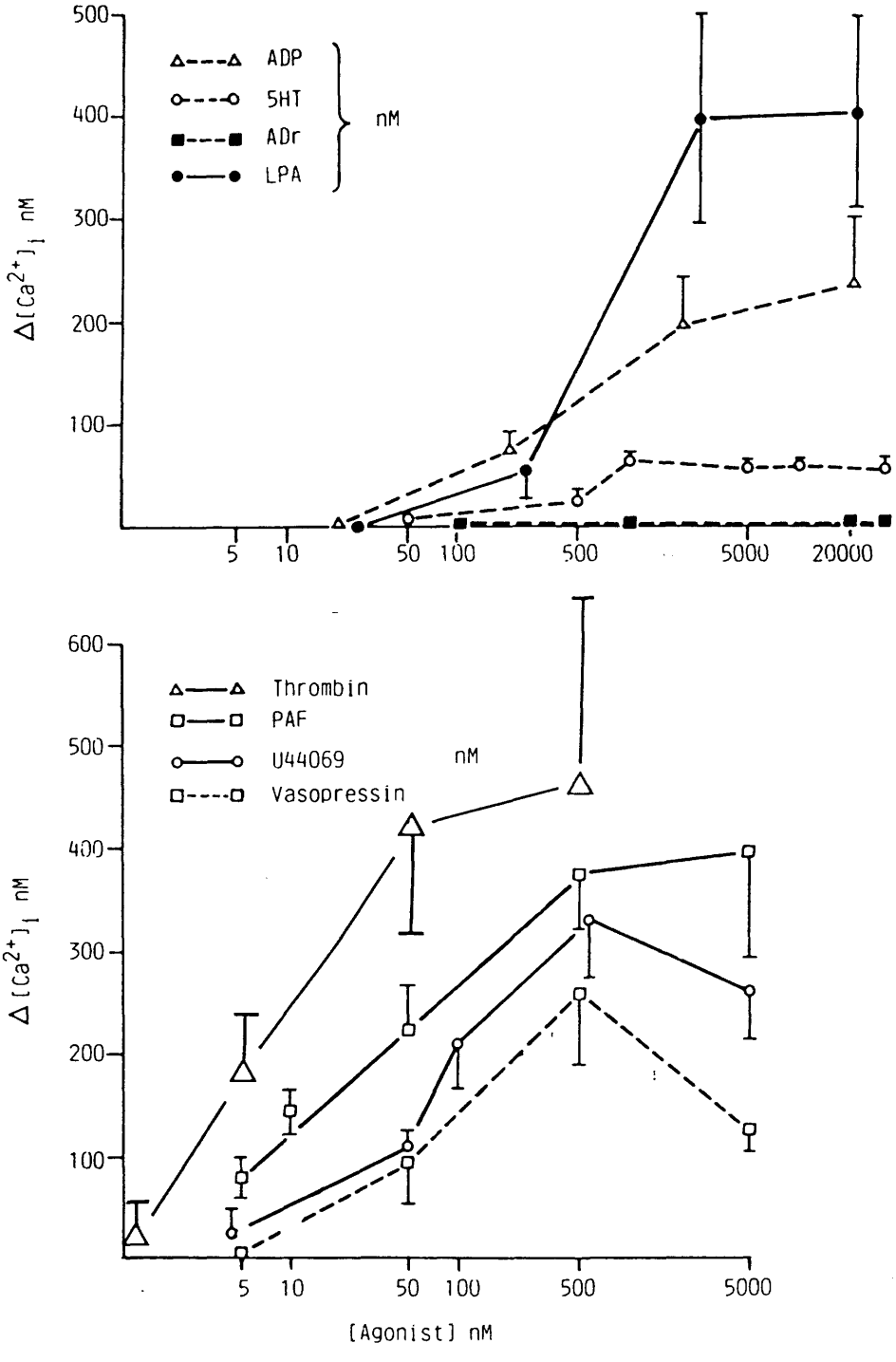


Figure 17. Agonist-induced elevation in cytosolic free Ca++ ([Ca++] i). Agonists , at the concentrations indicated, were added to Quin2-labelled platelets suspended in a medium of external free Ca++ = 1 mM. Changes in [Ca++] i were calculated from the observed changes in dye fluorescence. Results shown are the cumulative data from 3 - 9 experiments using platelets from different donors.

7.1. THE EFFECT OF PLATELET AGONISTS ON CYTOSOLIC FREE CALCIUM ($[Ca^{++}]_i$).

The aim of this study was to examine the effects of PAF, thrombin, U44069, LPA, vasopressin (VP), ADP, 5HT and adrenaline on platelet $[Ca^{++}]_i$.

$[Ca^{++}]_i$ in resting platelets was 90 ± 3 nM (means ± SE; n = 46). With the exception of adrenaline all agonists induced a concentration dependent elevation in $[Ca^{++}]_i$ (fig. 17). Threshold values and mean $E.C._{50}$ values (± S.E.) for agonist induced elevation of $[Ca^{++}]_i$ were as follows:- thrombin : 0.5 nM & 5 ± 2 nM; PAF : 5 nM & 50 ± 18 nM; U44069 : 10 nM & 80 ± 23 nM; LPA : 100 nM & 700 ± 300 nM; VP : 10 nM & 100 ± 40 nM; ADP : 100 nM & 500 ± 100 nM; 5HT : 300 nM & 700 ± 200 nM, respectively.

Thus the rank order of potency was thrombin > PAF > U44069 > vasopressin > ADP LPA > 5HT >> adr.

However the various agonists did not all elevate $[Ca^{++}]_i$ to the same extent: PAF, thrombin, U44069, LPA and vasopressin produced a maximum elevation of around 400 - 700 nM above basal whereas ADP and 5HT were less efficacious.

7.2. SOURCES OF CALCIUM

Having established that interaction with a receptor for most agonists presumably on the platelet plasma membrane, results in an elevation in $[Ca^{++}]_i$, the potential source of $[Ca^{++}]_i$ was examined.

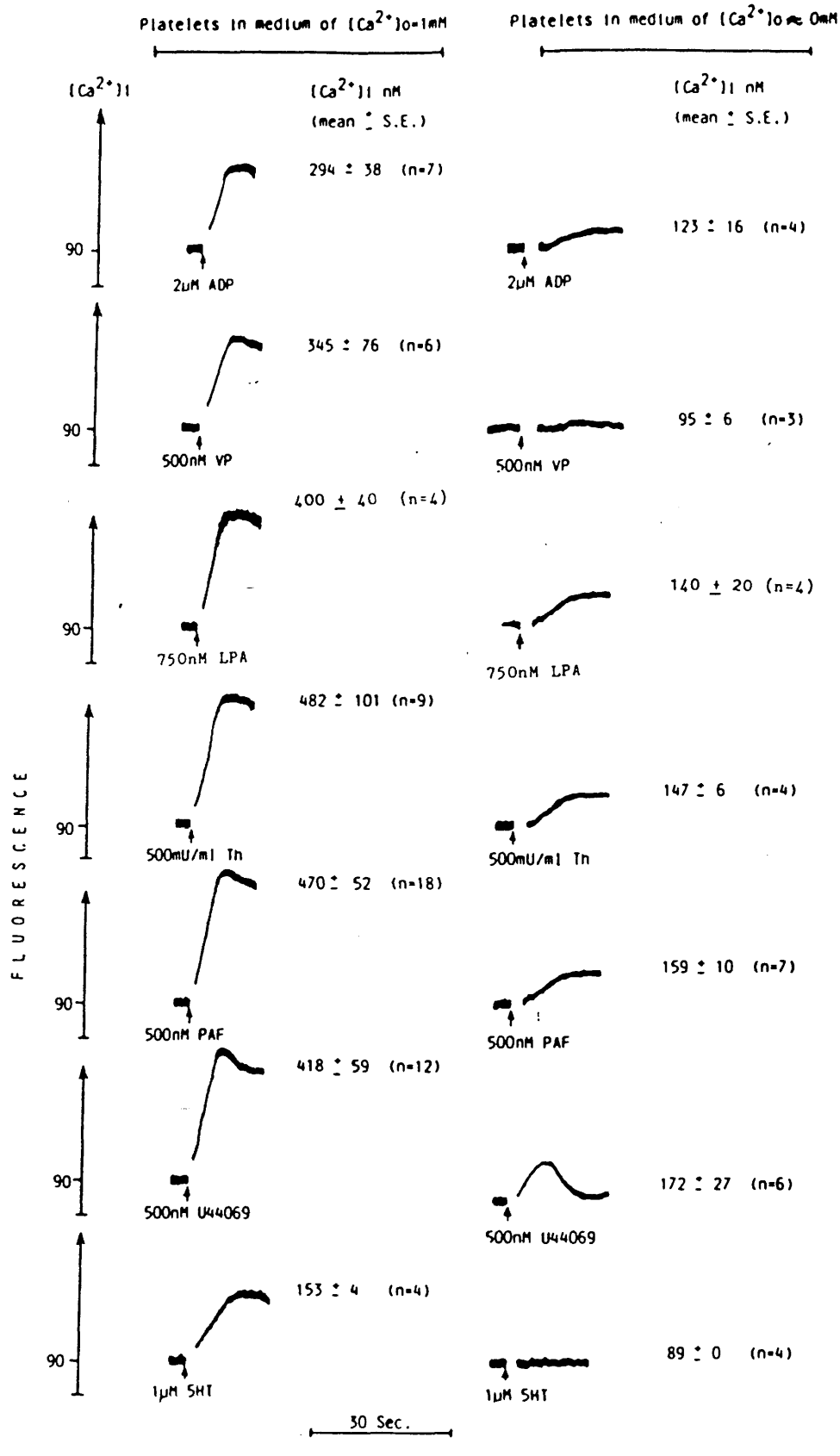


Figure 18. The effect of external Ca++ on agonist-induced elevations of [Ca++] i in platelets. Agonists at the concentrations indicated were added to quin2 loaded platelets suspended in normal medium (external free Ca++ = 1 mM) or in medium containing 3 mM EGTA (external free Ca++ = 0 mM). Changes in [Ca++] i were calculated from the observed changes in dye fluorescence. Results are typical fluorescence records for each individual agonist and mean values from replicate experiments performed using platelets from different donors.

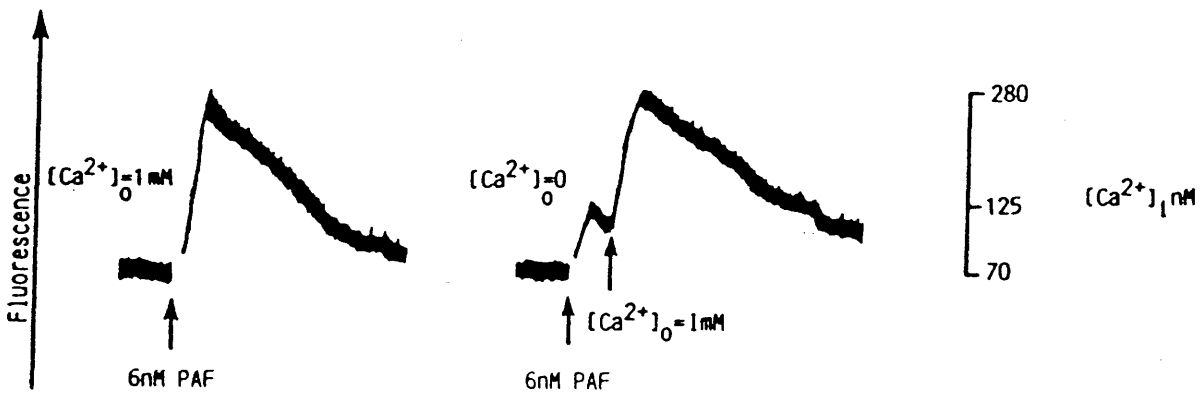


Figure 19. [Ca++]i originating from mobilization and influx. Quin-2 loaded platelets were stimulated with PAF. Fluorescence was monitored with time and shows the response recorded initially without adjusting [Ca++]o to 1mM (right; mobilization) and the effect of restoring [Ca++]o to 1mM (influx) after platelet activation. The normal response induced by PAF in the presence of [Ca++]o = 1mM is shown on the left. Changes in [Ca++]i were calculated from observed changes in dye fluorescence. Results are from a single observation and are representitive of at least 4 similar experiments using platelets from different donors.

Agonist-induced elevations in $[Ca^{++}]_i$ were monitored in the presence and absence of EGTA (3 mM) to chelate extracellular Ca++. In the presence of EGTA, where $[Ca^{++}]_o$ (extracellular free Ca++) = 0 (nominally zero), any changes in $[Ca^{++}]_i$ presumably are due to mobilization of intracellular Ca++. When $[Ca^{++}]_o = 0$, PAF, thrombin, U44069, LPA and ADP induced a concentration-dependent elevation in $[Ca^{++}]_i$ (see also fig. 22). The observed elevation in $[Ca^{++}]_i$ evoked by these agonists was approximately 20 % of the response recorded when $[Ca^{++}]_o$ = 1 mM. (Fig 18) In contrast 5HT and vasopressin did not elevate $[Ca^{++}]_i$ in the absence of extracellular Ca++.

An alternative approach to determine the possible source of $[Ca^{++}]_i$ may be adopted. When Quin2 labelled platelets are suspended in buffer containing no added Ca++ or EGTA (to facilitate the manipulation of $[Ca^{++}]_o$) addition of an agonist evokes an elevation in $[Ca^{++}]_i$ which rapidly reaches a peak then returns to the resting level. If $[Ca^{++}]_o$ is then adjusted to 1 mM a second elevation in $[Ca^{++}]_i$ is observed. The initial elevation in $[Ca^{++}]_i$ can be attributed to mobilization of intracellular Ca++ and the second to an influx of extracellular Ca++. Using platelets suspended in nominally Ca++ free medium, PAF induced an elevation in $[Ca^{++}]_i$ (fig. 19), when $[Ca^{++}]_o$ was adjusted to 1 mM immediately following the peak of the initial response, a futher elevation in $[Ca^{++}]_i$ was observed. The initial elevation in $[Ca^{++}]_i$ accounted for approximately 20 % of the total response observed.

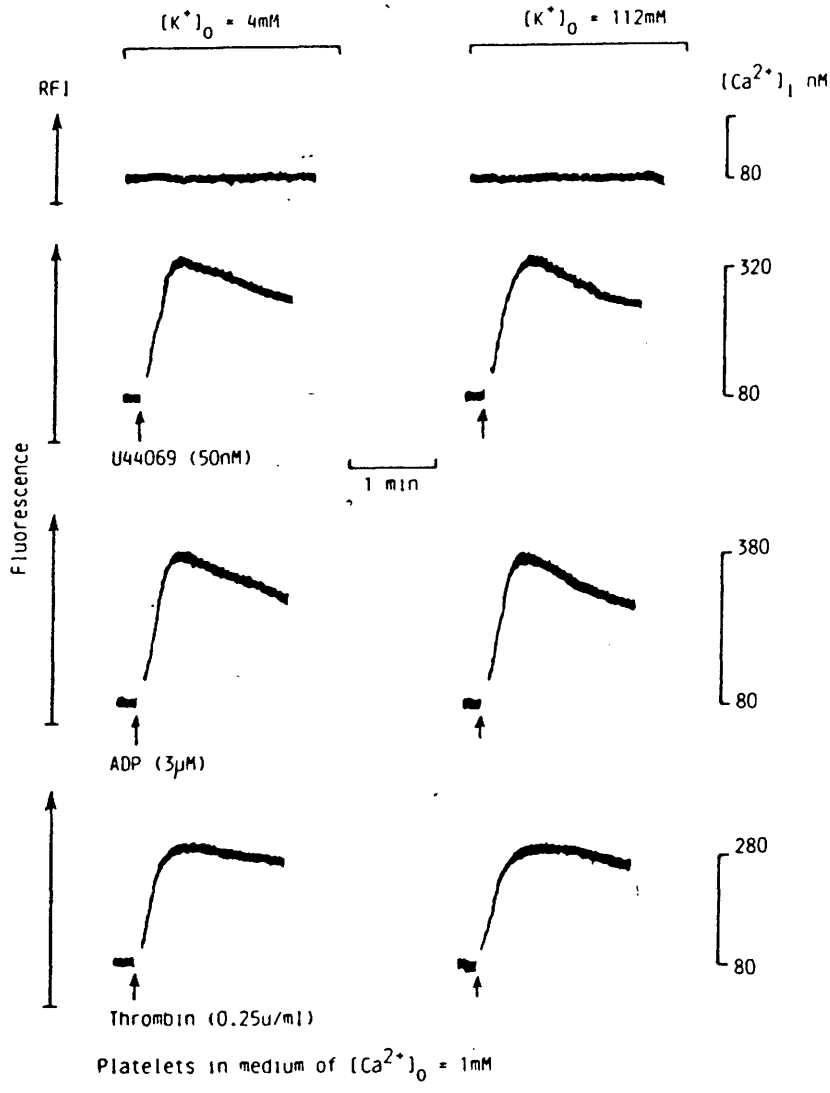


Figure 20. The effect of membrane depolarization on [Ca++] i and agonist-induced changes in [Ca++] i. Quin-2 loaded platelets were suspended in medium containing [K] o = 4mM (normal environment) and [K] o = 112mM to depolarize the membrane. In the latter situation osmolarity was maintained by adjusting Na+. Fluorescence was recorded immediately after depolarization and after the addition of thrombin, U44069 or ADP. Changes in [Ca++] i were calculated from observed changes in dye fluorescence. Results are taken from a single observation and are representative of 3 similar experiments using platelets from different donors.

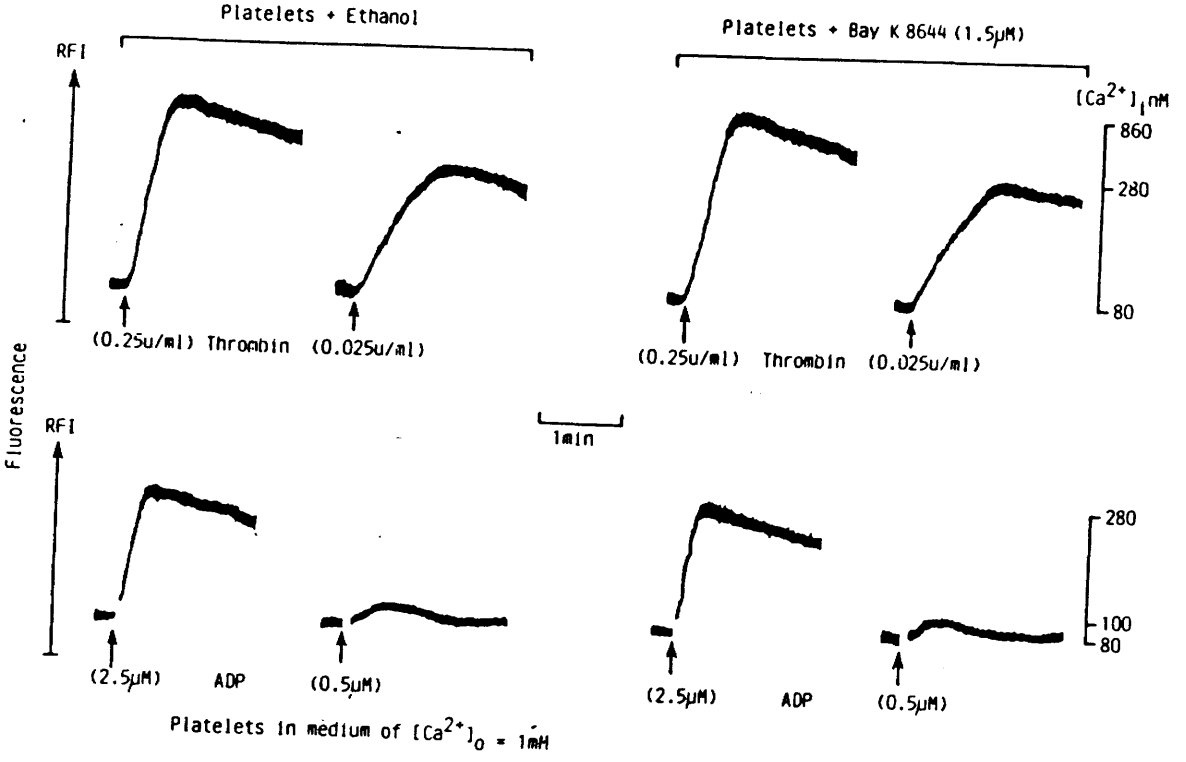


Figure 21. The effect of BAY K8644 on agonist-induced changes of [Ca++] i. BAY K8644 or vehicle in controls was preincubated for 2 minutes before the addition of thrombin or ADP at the concentrations indicated. Changes in [Ca++] i were calculated from the observed changes in dye fluorescence. Results are taken from a single experiment and are representative of 3 similar experiments using platelets from different donors.

7.3. THE EFFECTS OF MEMBRANE DEPOLARIZATION AND THE Ca++ AGONIST BAY K8644 ON RESTING $[Ca^{++}]_i$ AND AGONIST INDUCED $[Ca^{++}]_i$.

This study sought to establish the type of Ca++ channel present in the human platelet plasma membrane. VOC's may be selectively operated by the Ca++-agonist BAY K8644 or by depolarization of the membrane. The effect of membrane depolarization on $[Ca^{++}]_i$ was examined by suspending Quin2 loaded platelets in a medium containing $[K]_o$ (extracellular K concentration) = 4 mM (normal environment) and $[K]_o$ = 112 mM, which induces a considerable depolarization of the membrane.

Resting $[Ca^{++}]_i$ was unaltered by depolarization and no potentiation of the elevation in $[Ca^{++}]_i$ induced by thrombin, U44069 and ADP was observed (fig. 20)

BAY K8644 had no effect on resting $[Ca^{++}]_i$ (fig. 21). Since the action of the dihydropyridines are enhanced by membrane depolarization one would predict that increased $[Ca^{++}]_i$, evoked by agonists (thrombin & ADP) which allegedly produce membrane depolarization, would be potentiated in the presence of BAY K8644. Elevation of $[Ca^{++}]_i$ induced by low or high concentrations of thrombin or ADP was unaffected by BAY K8644 (fig. 21).

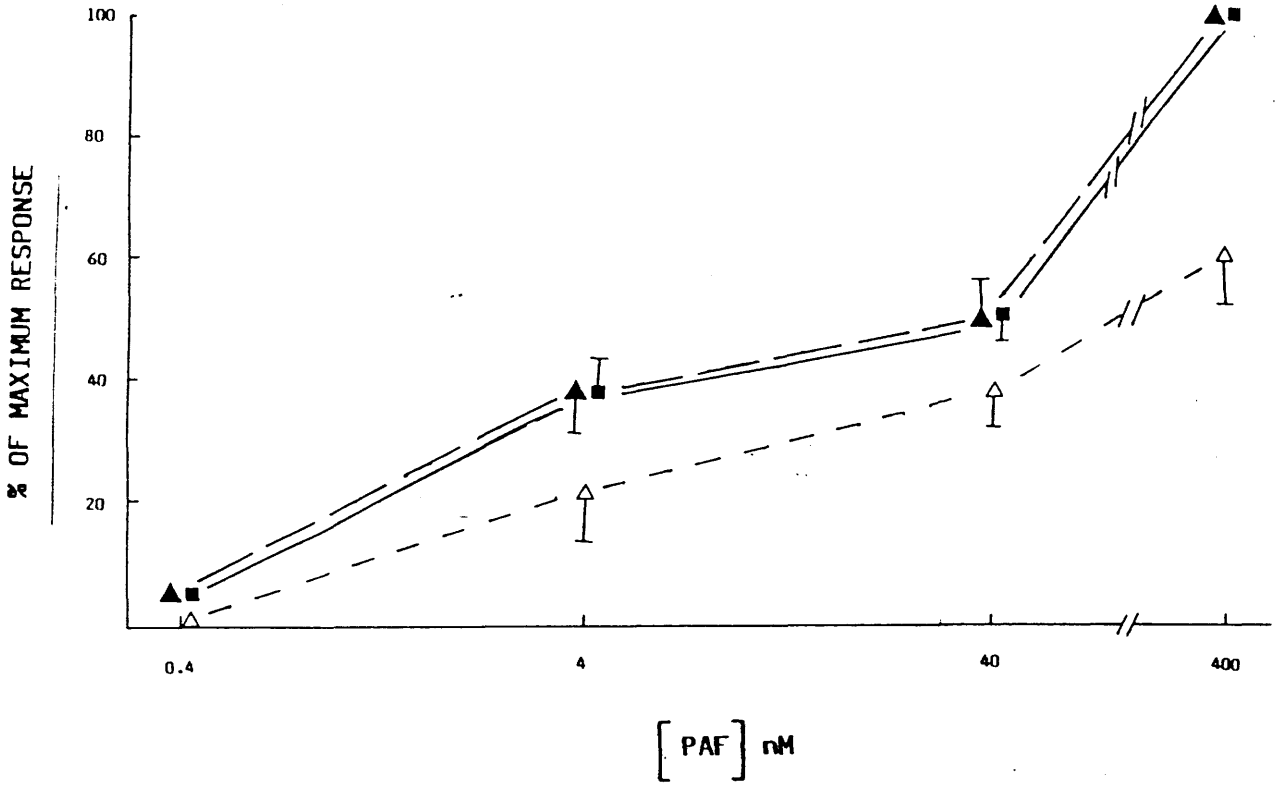


Figure 22. The effect of flurbiprofen and BW755C on PAF-induced changes in [Ca++] i. Flurbiprofen (100 μM) (▲—▲) or BW775C (100 μM) (Δ---Δ) or vehicle in controls (■— —■) was preincubated with quin2 loaded platelets for 3 minutes. Thereafter PAF, at the concentrations indicated on the abscissa, was added and the changes in fluorescence recorded, from which changes in [Ca++] i were calculated. The ordinate shows the response recorded as a % of the maximum response measured in the absence of drugs. The results are the mean values ± S.E. from three experiments using platelets from different donors.

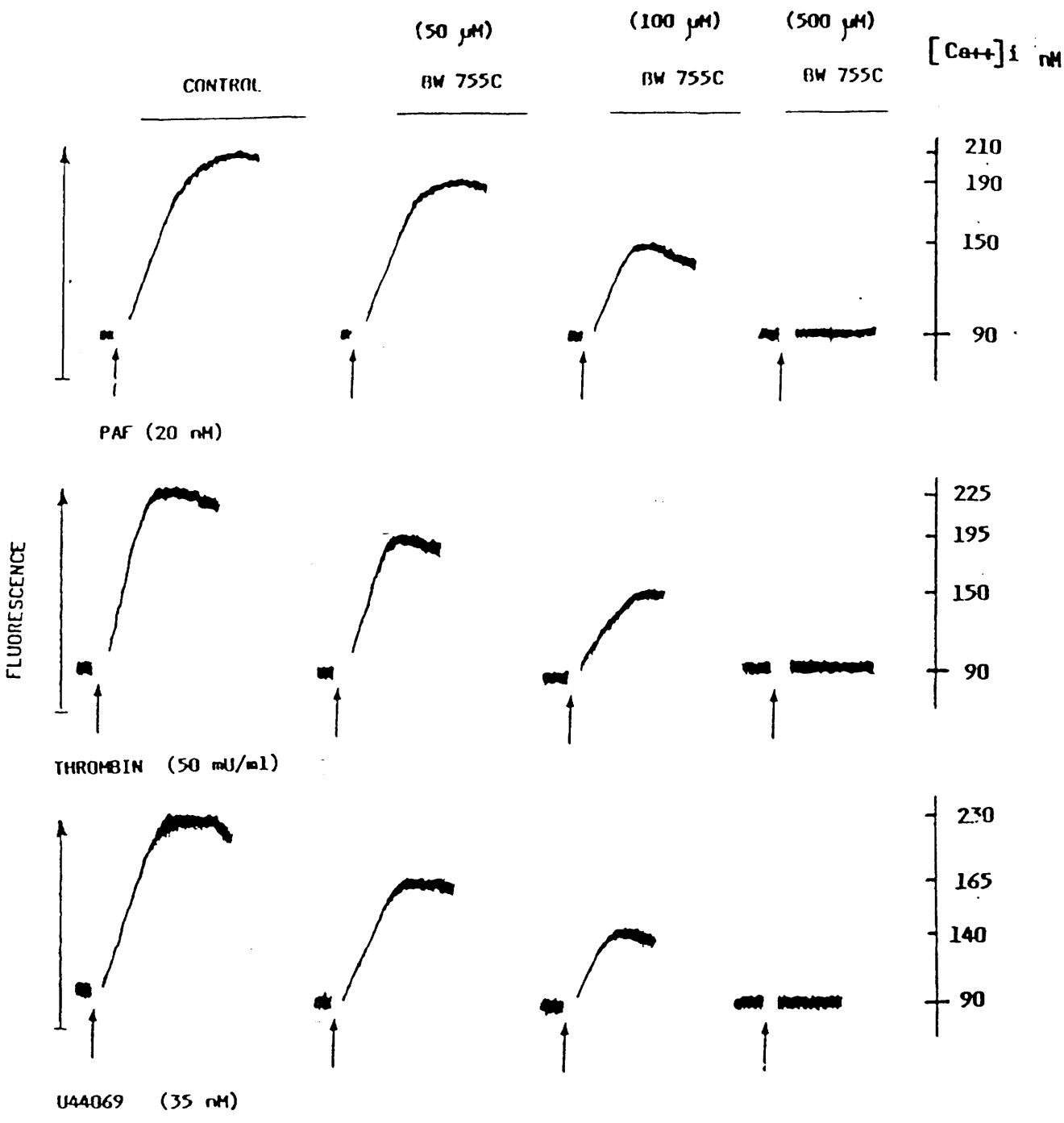


Figure 23. The effect of increasing concentrations of BW755C on elevations of [Ca++] i induced by submaximal concentrations of PAF, U44069 or thrombin. BW755C (or vehicle in controls) at the concentrations indicated was preincubated with quin2 labelled platelets. Agonists were added and changes in fluorescence recorded from which changes in [Ca++] i were calculated. The results are from a single experiment and are representative of 3 - 4 experiments using platelets from different donors.

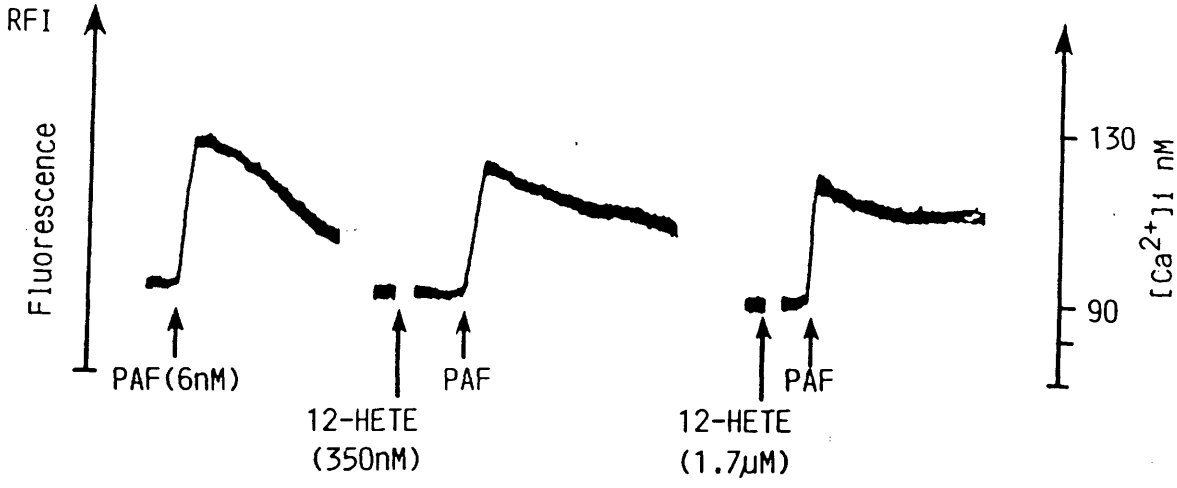


Figure 24. The effect of 12-HETE on PAF-induced changes in [Ca++] i. 12-HETE or vehicle (ethanol) in controls was preincubated with quin2 loaded platelets for 2 minutes. PAF at the concentrations indicated was added and the changes in fluorescence recorded. The results are taken from a single experiment and are typical of 3 similar experiments conducted using platelets from differend donors.

7.4. THE EFFECT OF CYCLOOXYGENASE INHIBITION, CYCLOOXYGENASE/LIPOXYGENASE INHIBITION AND OF 12-HETE ON PAF-INDUCED CHANGES IN CYTOSOLIC FREE CALCIUM.

To determine the effect of cyclooxygenase (CO) or cyclooxygenase/lipoxygenase (LO) inhibition inhibition on PAF-induced changes in $[Ca^{++}]_i$. Concentration response curves for changes in $[Ca^{++}]_i$ induced by PAF were constructed in the presence and absence of flurbiprofen, a competitive CO inhibitor, and BW755C, a CO/LO inhibitor.

Flurbiprofen at concentrations (100 μM) which inhibited aggregation induced by arachidonic acid had no effect on PAF-induced changes in $[Ca^{++}]_i$. In contrast BW755C (100 μM) inhibited PAF-induced elev tions in $[Ca^{++}]_i$. (fig. 22). However BW755C produced a concentration-dependent inhibition of PAF-induced elevation in $[Ca^{++}]_i$, and at high concentrations abolished the response. Responses evoked by agonists other than PAF, eg thrombin and U44069 were similarly affected (fig. 23), suggesting that the inhibitory effect of BW755C is non-specific.

Figure 24 shows that elevation of $[Ca^{++}]_i$ induced by low or high concentrations of PAF was unaffected by pretreating the cells with 12-HETE when compared to the vehicle control. In parallel experiments using washed platelets PAF-induced primary aggregation was also unaffected by prepreatment with 12-HETE (data not shown).

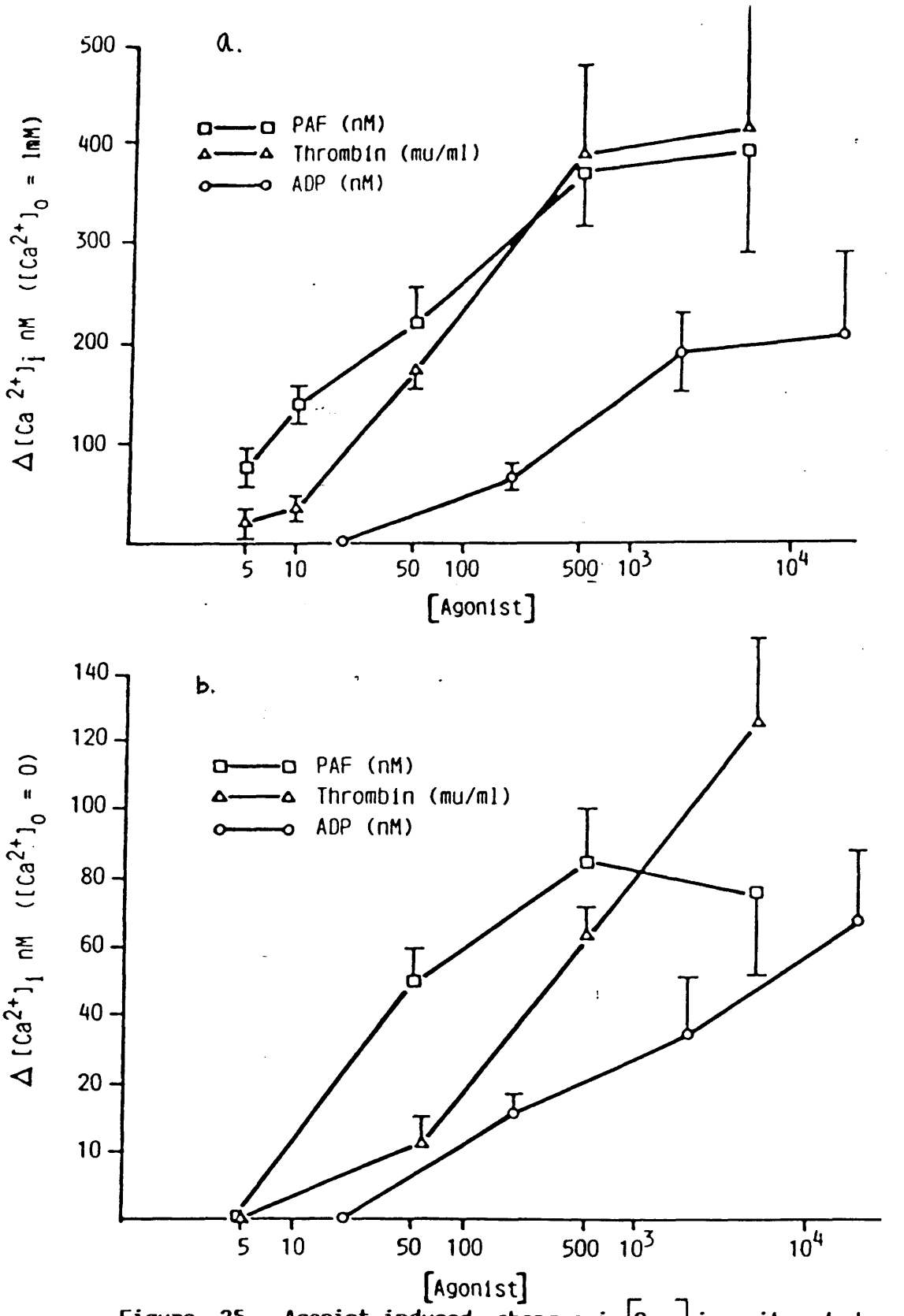


Figure 25. Agonist-induced changes in [Ca++] i monitored when [Ca++] o = 1 mM or [Ca++] o = 0 mM. Platelets were simultaneously labelled with quin2 and [^{14}C]-5HT. [Ca++] o was restored to 1 mM free or 0 mM by the addition of EGTA (3 mM). Thrombin, PAF or ADP, at the concentrations indicated on the abscissa, were added and fluorescence changes recorded. Reactions were terminated immediately after the maximum change in fluorescence by the addition of 1 vol. EGTA/imipramine and the released [^{14}C]-5HT measured. Agonist-induced changes in [Ca++] i when [Ca++] o = 1 mM or 0 mM are shown in figure 25 whilst the corresponding [^{14}C]-5HT release is shown in figure 26. Results are the mean values ± S.E. from 3 - 4 experiments.

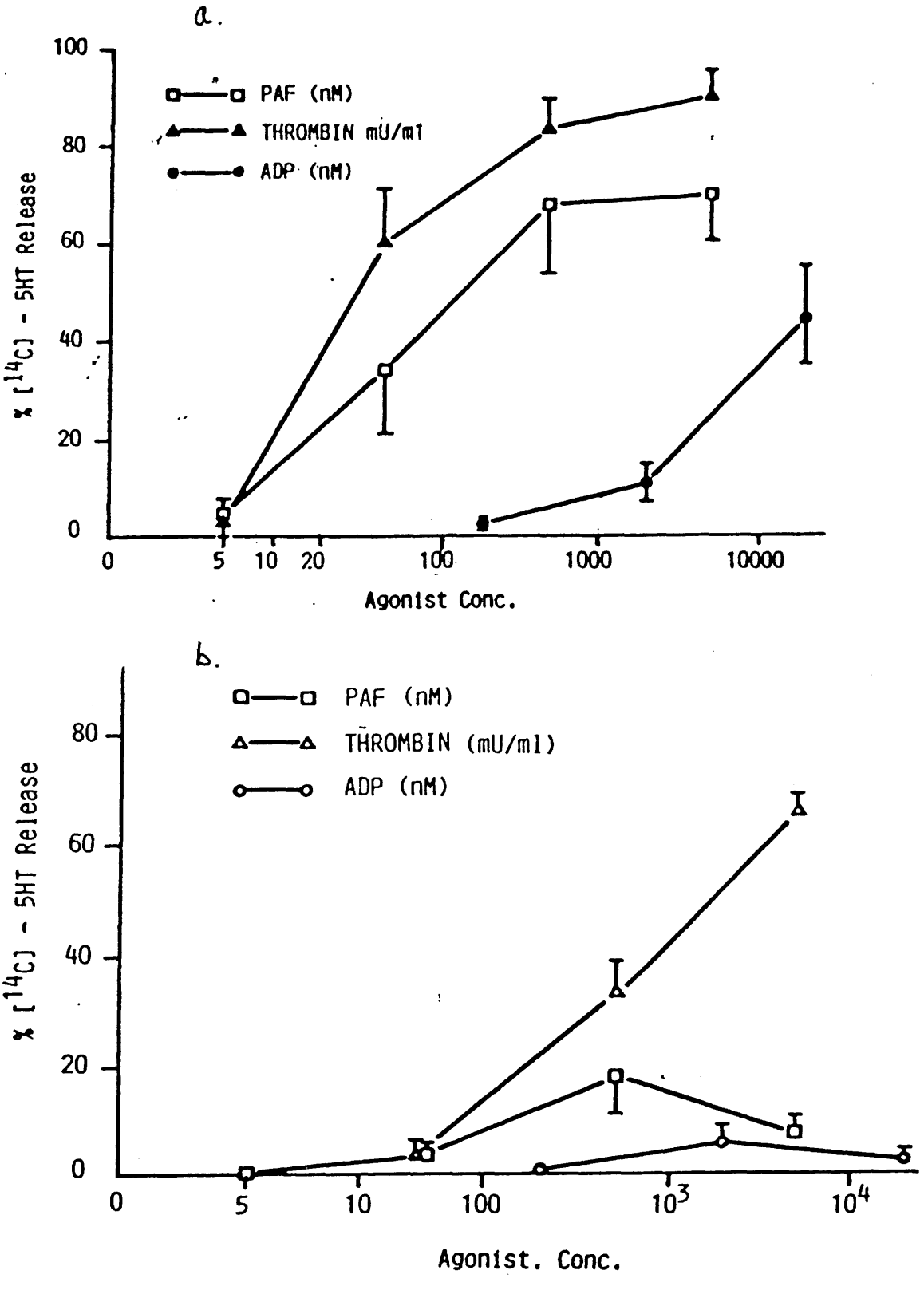


Figure 26. Agonist-induced $[^{14}C]$-5HT release monitored when $[Ca^{++}]_o$ = 1 mM or $[Ca^{++}]_o$ = 0 mM. Protocol is described in figure 25.

7.5. CORRELATION BETWEEN $[Ca^{++}]_i$ AND PLATELET FUNCTIONAL RESPONSE.

Calcium is required as an essential cofactor in many biochemical reactions underlying platelet functional responses. A generalized elevation in $[Ca^{++}]_i$ might satisfy these requirements. However agonists induce a concentration-dependent elevation in $[Ca^{++}]_i$, supporting the hypothesis that this ion truely mediates cellular response. This being so, it should be possible to establish a direct correlation between $[Ca^{++}]_i$ and some functional response.

PAF, thrombin and ADP evoke a concentration-dependent elevation in $[Ca^{++}]_i$ (fig. 25 a). When $[^{14}C]$-5HT release was monitored in parallel, as a measure of functional response, all three agonists induced a concentration-dependent release of $[^{14}C]$-5HT (fig. 26 a). However, although PAF and thrombin evoked a similar maximum elevation in $[Ca^{++}]_i$, maximum $[^{14}C]$-5HT release induced by thrombin (88 $\pm$ 6 %) was significantly greater than that induced by PAF (68 $\pm$ 5 %). ADP, which does not elevate $[Ca^{++}]_i$ to the same extent as PAF or thrombin induced a maximun $[^{14}C]$-5HT release of 43 $\pm$ 10 % (means $\pm$ S.E. from 3 - 4 experiments conducted in duplicate). Moreover the elevation in $[Ca^{++}]_i$ produced by 2 μM ADP (200 nM) was similar to that produced by 50 mU/ml thrombin and 50 nM PAF yet $[^{14}C]$-5HT release induced by ADP was 10 % compared with 33 % and 60 % for PAF and thrombin respectively.

When extracellular calcium is chelated using EGTA, the elevation in $[Ca^{++}]_i$ induced by PAF thrombin and ADP was

inhibited by 80 % (fig. 25 b). Under these conditions , when compared to controls where $[Ca^{++}]_o$ = 1 mM, the maximum $[^{14}C]$-5HT release induced by PAF (17 $\pm$ 5 %) was inhibited by 75 %, and that induced by ADP (6 $\pm$ 2 %) was inhibited by 85 %, however, although the concentration response curve for thrombin was displaced to the right the maximum release induced by thrombin (65 %) was only inhibited by 25 % (fig. 26 b).

CONCLUSION

The hypothesis that $[Ca^{++}]_i$ may act as a stimulus-response coupling agent was initially drawn from analogy with other secretory systems **(Rubin, 1979)**. The discovery of the protein actomyosin in platelets **(Bettex-Galland & Luscher 1961)** reinforced this hypothesis since a regulatory role for Ca^{++} was well established in stimulus-contraction coupling in skeletal, cardiac and smooth muscle **(Taylor & Godt, 1976; Fleckenstein, 1971;Breeman, 1973)**. Experiments using the Ca^{++} ionophore A23187 indirectly inferred a central role for $[Ca^{++}]_i$ in stimulus-response coupling in platelets and suggested that platelets could utilize both intracellular and extracellular calcium. Studies designed to measure the uptake of $^{45}Ca^{++}$ are complicated, both technically and in the interpretation of results. For example **Owen et al. (1980)** found that adrenaline but not ADP induced a concentration-dependent uptake of $^{45}Ca^{++}$ which correlated with aggregation. **Massini & Luscher (1976)** also observed uptake of $^{45}Ca^{++}$ in response to thrombin stimulation, but unlike Owen and colleagues they attributed this to an exchange reaction occuring concomitantly with the release reaction and could be prevented by inhibitors of the release reaction. Results using the Ca^{++} indicator dye chlortetracycline (CTC) also suggest a positive relationship between $[Ca^{++}]_i$ and platelet activation. However, as noted in the section 3, there is some ambiguity over the intepretation of CTC results

The Quin-2 system recorded a $[Ca^{++}]_i$ in resting platelets of around 10^{-7} nM: a value in good accord with resting values quoted for other cell types (eg. smooth muscle) recorded by conventional methods (**Breeman, 1973**) and that recorded in platelets using null-point determination (**Pudon et al, 1984**). With the exception of adrenaline, which was inactive, all agonists induced a concentration-dependent elevation of $[Ca^{++}]_i$. Moreover ADP and 5HT, considered to be weak platelet agonists, were incapable of elevating $[Ca^{++}]_i$ to the same extent as the powerful agonists such as thrombin or PAF. Such results tentatively suggest that Ca++ may indeed act as the second messenger that mediates the cellular responses initiated by occupancy of receptors for thrombin, PAF, U44069, LPA, vasopressin, ADP and 5HT on the platelet.

The anomalous result obtained using adrenaline clearly complicates this hypothesis however as discussed in section 3.3., a reduction in the intraplatelet level of cAMP may also serve as a transduction process leading to activation of platelets. This may be the mechanism by which adrenaline acts, since adrenaline has been reported to lower an elevated platelet cAMP level (**Salzman, 1974**). Although inhibition of adenylate cyclase as a mechanism for platelet activation is currently the subject of much controversy, alpha-2 adrenoceptors must initiate platelet activation by mechanisms unrelated to changes in $[Ca^{++}]_i$ (at least as monitored using Quin2).

A number of workers (**Massini & Luscher, 1974; Feinman et al.,**

1974; White et al., 1974) have reported that the Ca++-ionophore A23187 can induce platelet activation not only when $[Ca^{++}]_o$ = 1 mM but also when extracellular Ca++ has been chelated, implying that these cells can utilize Ca++ of both external and internal origin. **Le Breton et al., (1982)** have suggested that the origin of $[Ca^{++}]_i$ may in fact depend on the agonist. These workers reported that both ADP and interestingly, adrenaline caused a decrease in CTC fluorescence, indicating an increase in $[Ca^{++}]_i$. The decrease in CTC fluorescence induced by ADP but not by adrenaline was shown to be sensitive to D_2O. In parallel studies adrenaline but not ADP increased the uptake of $^{45}Ca^{++}$. These results were interpreted as indicating that adrenaline activated platelets by causing an influx of extracellular Ca++ whereas ADP promoted mobilization of intracellular Ca++ only **(Le Breton et al. 1982).**

In this study, when the possible sources of $[Ca^{++}]_i$ were examined using platelets suspended in medium of $[Ca^{++}]_o$ = 0 mM, PAF, thrombin and ADP evoked a concentration-dependent elevation in $[Ca^{++}]_i$ which accounted for 20 % of the response recorded when $[Ca^{++}]_o$ = 1 mM. These results suggest that, for these agonists; elevation of $[Ca^{++}]_i$ is composed of two components: the principle component being an influx of extracellular Ca++, and the minor component consisting of mobilization of intracellular Ca++ ($\geq$ 20 % of the total signal). Whether the normal response is composed of both components (ie. influx and mobilization) remains to be proven. Vasopressin and 5HT, in contrast, only appear to be capable of

inducing Ca++ influx.

Since neither membrane depolarization nor the Ca++-agonist BAY K8644 altered resting $[Ca^{++}]_i$ or potentiated agonist-induced elevation of $[Ca^{++}]_i$, the inward Ca++ current produced by the agonists examined appears to be carried through receptor operated calcium channels.

As inhibition of cyclooxygenase had no effect on PAF-induced changes in $[Ca^{++}]_i$ it must be concluded that PGG_2, PGH_2 & TXA_2 do not mediate PAF-induced dense body secretion by increasing $[Ca^{++}]_i$. Although BW755C inhibited agonist-induced changes in $[Ca^{++}]_i$ this effect occured at high concentrations and was apparently non-specific. Moreover although low concentrations of U44069 are reported to induce arachidonic acid release, the free acid is not metabolised by LO or CO **(Siess et al., 1985)**. This being so then the inhibitory effect of BW755C cannot be mediated through inhibition of cyclooxygenase or lipoxygenase.

Although a concentration dependent elevation in $[Ca^{++}]_i$ has been established for the agonists examined, this does not prove a causal relationship between $[Ca^{++}]_i$ and functional response. Therefore in the last study of this section an attempt was made to correlate $[Ca^{++}]_i$ with secretion of dense body constituents by examining the concentration response relationships for both events.

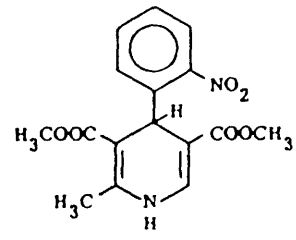
With any individual agonist a direct correlation was observed between $[Ca^{++}]_i$ and platelet functional response (at least when

$[Ca^{++}]_o$ = 1 mM). However for the same $[Ca^{++}]_i$ induced by different agonists (PAF, thrombin or ADP) the functional response did not correlate with $[Ca^{++}]_i$. In the absence of extracellular Ca++ $[Ca^{++}]_i$ induced by thrombin, PAF or ADP is inhibited by 80 %. Therefore one might predict that if the cytosolic free calcium concentration mediates the functional response then it (ie. functional response) would also be inhibited by 80 %. Indeed for PAF and ADP this was the case but only a small inhibition was observed with thrombin.

$[Ca^{++}]_i$ is clearly an important determinant of secretion of dense body constituents, at least for PAF and ADP. However equally apparent from these results is that platelet functional responses induced by thrombin do not correlate well with $[Ca^{++}]_i$, suggesting the involvement of other intracellular mediators (eg. 1,2-diacylglycerol; **Nishizuka 1984**) see section 4.

8. PHARMACOLOGICAL MANIPULATION OF THE CYTOSOLIC FREE CALCIUM CONCENTRATION: CALCIUM CHANNEL BLOCKERS

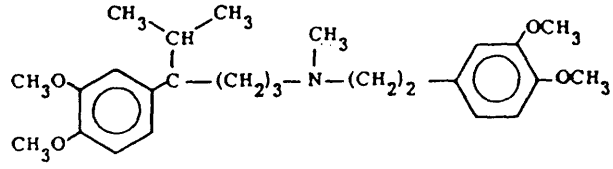
Class I: (a) 1,4-Dihydropyridines e.g. Nifedipine



(b) Flunarizine

(c) Fendiline

Class II: Phenylalkylamines e.g. Verapamil



Class III: Benzothiazepines e.g. Diltiazem

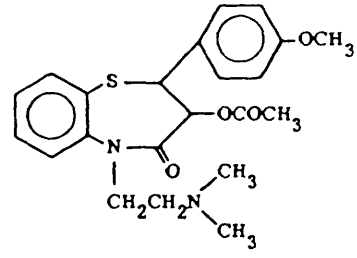
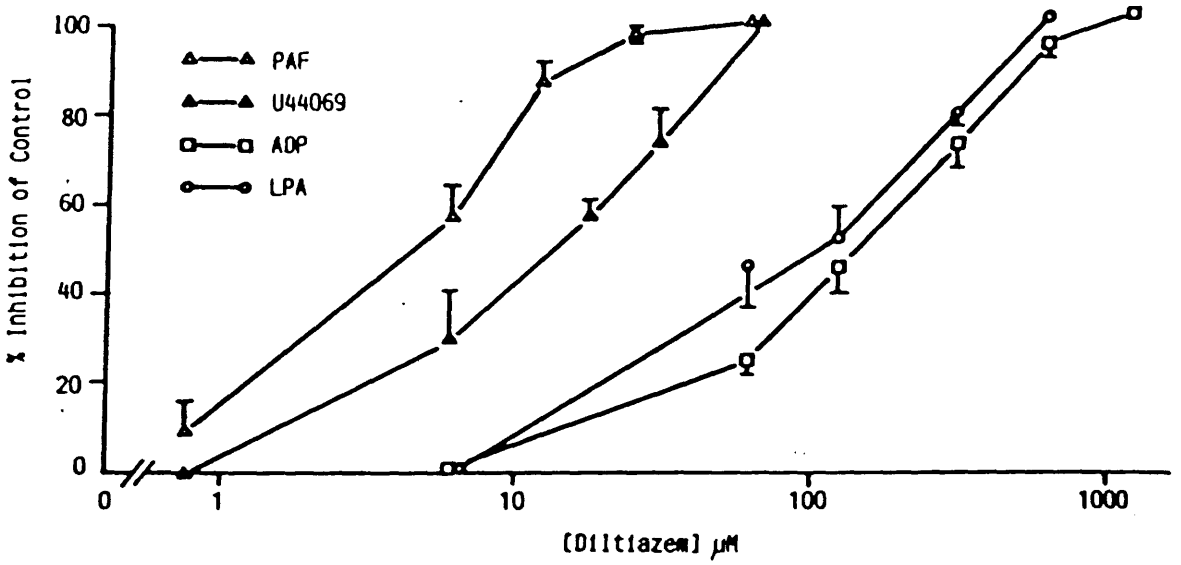


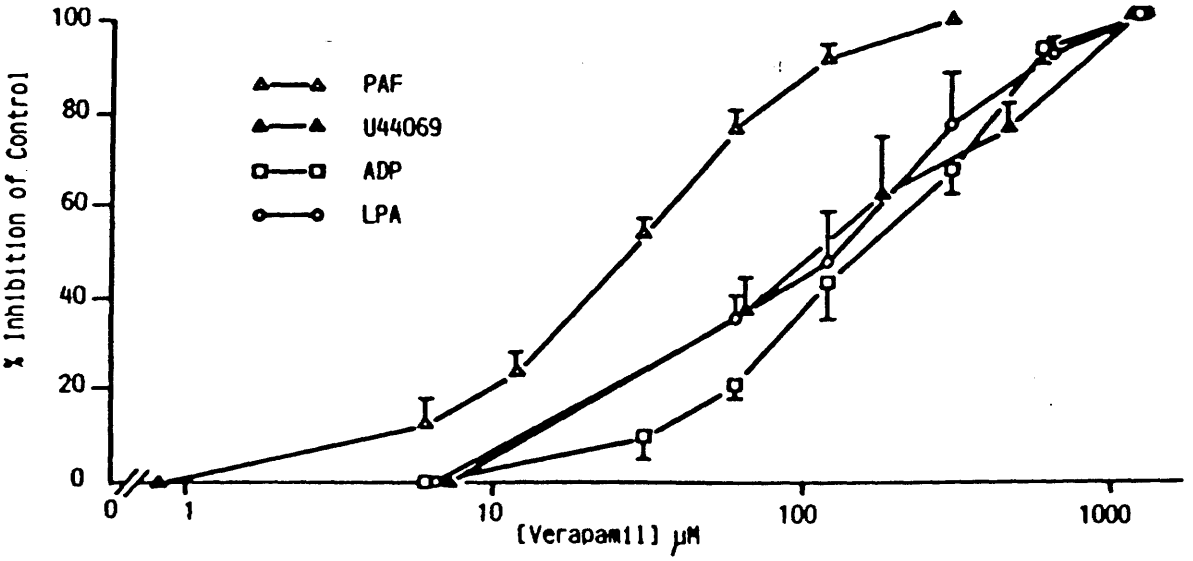
Figure 27. Classes of calcium channel blocker proposed by Glossman et. al (1982) from studies using [^{3}H]-nimodipine.

The results gleaned from section 7 have established that most agonists induce a concentration-dependent elevation in $[Ca^{++}]i$. In this section I examined the effects of drugs known to modify the availability of $[Ca^{++}]i$ on platelet functional response. The major component of $[Ca^{++}]i$ is an influx of extracellular Ca++ through a receptor operated Ca++-channel in the platelet plasma membrane. Calcium antagonists or calcium channel blockers are drugs that block the inward calcium current through VOC's and ROC's **(Cauvin et. al., 1983).** Recently on the basis of radioligand binding studies, using $[^{3}H]$-nimodipine **Glossman et. al. (1982)** have proposed three classes of calcium antagonist/calcium channel blocker (fig 27) : class 1 includes the 1,4 dihydropyridines, flunarizine and fendiline while the phenylalkylamines represent the principle agents in class II, and the benzothiazepines represents class III. In the first study I examined the effect of nicardipine **(class I),** verapamil **(class II)** and diltiazem **(class III)** on platelet aggregation induced by various agonists.

The terminology used for these drugs (ie. calcium antagonist/calcium channel blocker) has created much confusion and arguement. **Fleckenstein (1971)** introduced the term calcium-antagonist to describe the action of those drugs that specifically inhibit Ca++-dependent exitation-response coupling. Other synonyms include Ca++-blockers, slow channel blockers, slow channel antagonists and calcium entry blockers. In isolated heart cells **Tsien (Lee et al, 1983)** has demonstrated that the three classes (1,4 dihydropyridines;

phenylalkylamines; benzothiazepines) of calcium antagonist/calcium channel blocker block both the inward and outward ion currents in these cells, implying true channel blockade. Moreover he also shows that elevation of the extracellular calcium concentration can overcome the blockade by these drugs: an effect in accordance with Ca++-antagonism. Clearly, therefore, both calcium channel blocker or calcium antagonist may be appropriate terms. Strict interpretation of Ca++-antagonism implies an action at the site of the calcium receptor. However calcium channel blocker is also a rather ambiguous term in that it implies physical obstruction of the channel as the mechanism of action of these agents. **Glossman et al., (1982)** using Guinea pig brain membranes has shown that the 1,4 dihydropyridines bind to the outer mouth of the putative calcium channel whereas the phenylalkylamines bind to the inner mouth of the channel. Therefore it is likely that these two classes prevent the inward calcium current by different mechanisms. Since the primary interest of this study is the pharmacological effect of these agents, that is preventing an inward calcium current, this action is more appropriately described by calcium channel blockade rather than calcium antagonism hence the former term has been applied throughout these studies.


% Inhibition of Control
100
80
60
40
20
0
PAF
U44069
ADP
LPA
0
1
10
100
1000
[Diltiazem] μM


% Inhibition of Control
100
80
60
40
20
0
PAF
U44069
ADP
LPA
0
1
10
100
1000
[Verapamil] μM

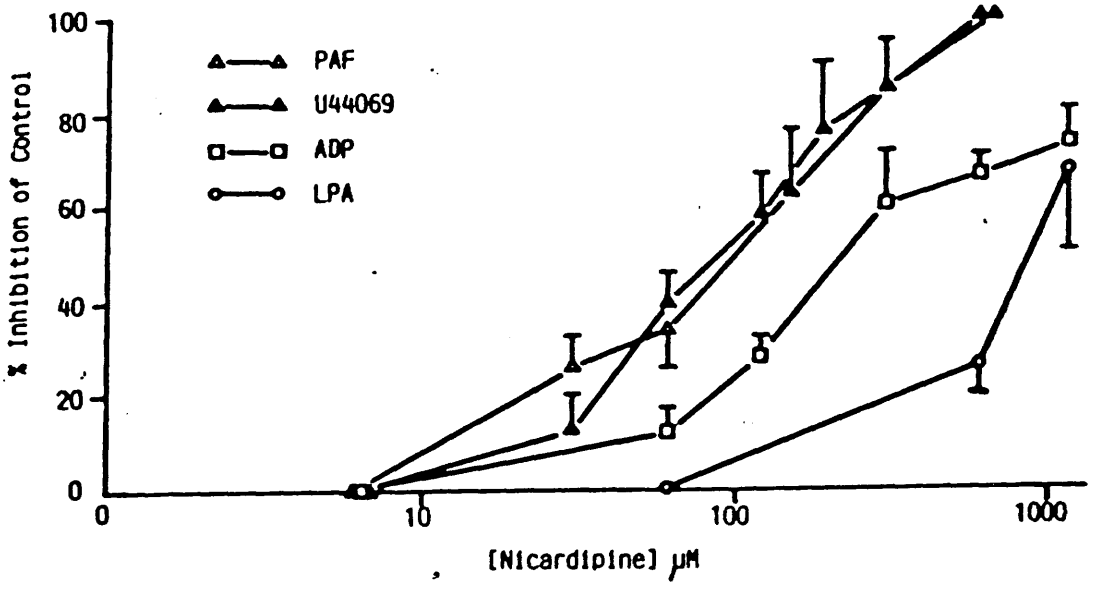


Figure 28. **Inhibition of human platelet primary aggregation by class I, class II and class III calcium channel blockers.** Increasing concentrations of nicardipine (class I), verapamil (class II) and diltiazem (class III) were incubated with aspirin treated PRP for 2 min. at 37 °c before the addition of agonists at concentrations which induce submaximal primary aggregation, normally 40 - 70% of the maximum response. Results are expressed as percentage inhibition of the control response (ie. vehicle in place of CCB) and are the mean values from 6 - 8 experiments.

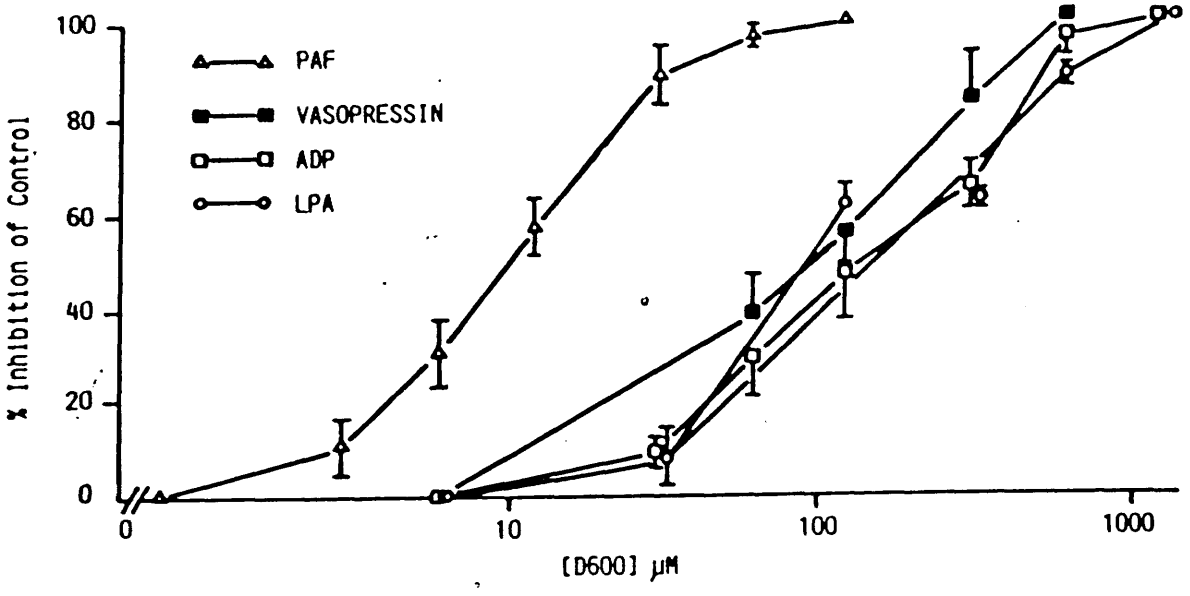


Figure 29.. Inhibition of platelet aggregation by methoxyverapamil. Experiments were performed as described for fig. (28) Results are the mean values ± SE from 6 experiments.

Calcium nnel Blocker	IC_{50} Value (μM) Versus Agonist:							
	PAF	(n)	U44069	(n)	LPA	(n)	ADP	(n)
cardipine Class I)	100±20	(8)	94±16	(5)	900±50	(4)	240±30	(6)
rapamil Class II)	*** 25±3	(11)	120±20	(4)	100±20	(6)	185±26	(8)
ltiazem Class III)	*** 5±1	(10)	*** 12±3	(6)	100±17	(10)	133±13	(10)

*** P 0·001 : Variance Ratio Test - PAF V U44069, PAF V LPA or PAF V ADP.

Table 3. The effect of calcium channel blockers on platelet aggregation. Concentration response curves for inhibition, by verapamil, nicardipine and diltiazem, of submaximum aggregation resposes induced by PAF, U44069, ADP and LPA were performed essentially as described for figure 28. The results show the mean IC_{50} values ± S.E. (concentration of C.C.B. which inhibited the agonist response by 50%) calculated from the IC_{50} value from 5 - 8 individual curves.

8.1. THE EFFECT OF CLASS I, CLASS II AND CLASS III CALCIUM CHANNEL BLOCKERS ON HUMAN PLATELET AGGREGATION.

Using PRP from aspirin treated donors, to obviate the involvement of endogenous platelet mediators, concentration response curves for inhibition, by nicardipine (class I), verapamil (class II) and diltiazem (class III), of submaximum aggregation (usually 40 % - 70 % of the maximum response) induced by PAF, ADP, U44069 and LPA (fig. 28) were constructed. From these curves IC_{50} values (the concentration that produced 50 % inhibition of the control value) were estimated (table 3).

8.2. IS THE SELECTIVE INHIBITION OF PAF-INDUCED PLATELET AGGREGATION RESTRICTED TO THE PHENYLALKYLAMINES?

Verapamil appears to be a selective inhibitor of aggregation induced by PAF. To establish whether this effect is common to the class II calcium channel blockers (CCBS) I examined the effect of methoxyverapamil (D600) on submaximal aggregation induced by PAF, vasopressin, ADP and LPA (fig. 29) and also the effect of other class I CCBS on aggregation induced by PAF. Methoxyverapamil was a more potent inhibitor of aggregation induced by PAF (IC_{50} = 12.5 $\pm$ 2.5 μM; n = 10) than verapamil (IC_{50} = 25 $\pm$ 3 μM) and was approximately ten fold less potent against aggregation induced by vasopressin (IC_{50} = 110 $\pm$ 21 μM; n = 4), ADP (IC_{50} = 180 $\pm$ 23 μM;

Calcium Channel Blocker	IC_{50} Value (μM) Versus Agonist:							
	PAF	(n)	U44069	(n)	LPA	(n)	ADP	(n)
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Verapamil (Class II)	*** 25±3	(11)	120±20	(4)	100±20	(6)	185±26	(8)
Diltiazem (Class III)	*** 5±1	(10)	*** 12±3	(6)	100±17	(10)	133±13	(10)

*** P 0·001 : Variance Ratio Test - PAF V U44069, PAF V LPA or PAF V ADP.

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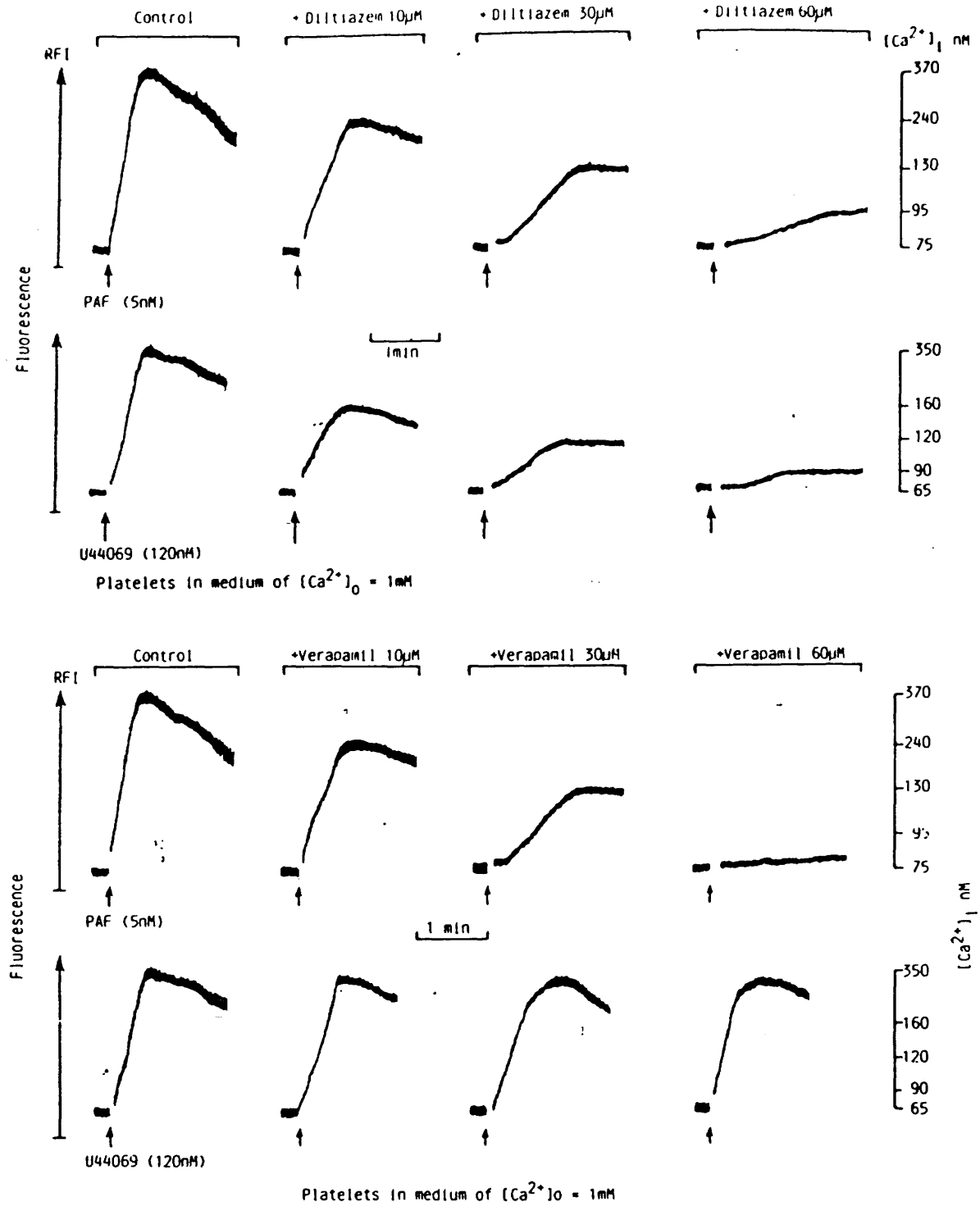

Figure 30. The effect of verapamil and diltiazem on [Ca++] i induced by PAF and U44069. Increasing concentrations of verapamil or diltiazem were incubated with Quin-2 loaded platelets, from aspirin treated donors, for 2 min. at 37°c before addition of PAF or U44069 at concentrations which induced submaximal fluorescence responses. The results were performed using platelets from a single donor and are typical of the results obtained from 4 similar experiments.

n = 4) and LPA (IC_{50} = 130 ± 30 μM; n = 8) (means ± SE) which were inhibited to a similar extent.

Flunarizine (IC_{50} = 700 μM), cinarizine (IC_{50} > 300 μM) and bepridil (IC_{50} = 250 μM), class 1 CCBS, only inhibited PAF-induced aggregation at high concentrations.

8.3. THE EFFECT OF VERAPAMIL AND DILTIAZEM ON ELEVATION OF $[Ca^{++}]_i$ INDUCED BY PAF AND U44069.

Verapamil and diltiazem inhibit PAF-induced aggregation. Only verapamil is selective for PAF, whereas diltiazem inhibits aggregation induced by both PAF and U44069. Accordingly if the inhibitory action of of these drugs is purely due to blockade of a receptor operated calcium channel then a similar pattern should be observed for inhibition of $[Ca^{++}]_i$.

The effect of verapamil and diltiazem was examined on similar submaximal elevations in $[Ca^{++}]_i$ induced by PAF and U44069 (fig. 30). Both drugs produced a concentration-dependent inhibition of $[Ca^{++}]_i$ induced by PAF. In contrast $[Ca^{++}]_i$ induced by U44069 was inhibited by diltiazem only.

8.4. THE EFFECTS OF VERAPAMIL ON CALCIUM MOBILIZATION AND CALCIUM INFLUX INDUCED BY PAF.

The selectivity of verapamil which was observed with aggregation induced by PAF, also extends to the elevation in cytosolic free calcium induced by PAF. If the CCBS act by preventing the inward Ca++ current, an action equivalent to

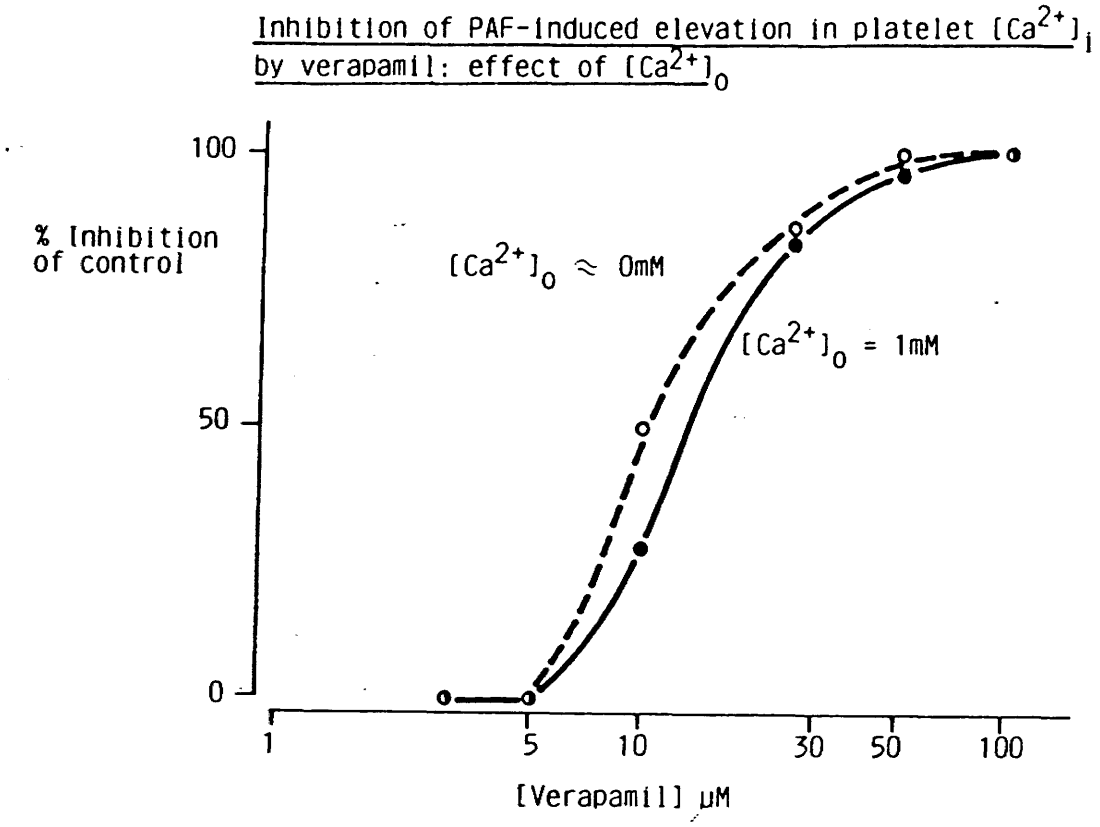

Figure 31. Inhibition of PAF-induced elevations in [Ca++] i by verapamil both when [Ca++] o = 1 mM and when [Ca++]o= 0 mM. Verapamil at the concentrations indicated on the abscissa were incubated with qiun2 labelled platelets ± EGTA (3 mM). The changes in fluorescence induced by submaximum concentrations of PAF were recorded and [Ca++] i calculated from the observed changes in fluorescence. The results are from one experiment and are typical of 4 similar experiments using platelets from different donors.

extracellular Ca++ deprivation, then one might expect a residual component of the agonist response (ie. 20 %), originating from intracellular locations, to be insensitive to these agents.

Verapamil (and diltiazem) apparently abolish total $[Ca^{++}]_i$ implying that these agents not only prevent influx but also prevent mobilization of intracellular Ca++. I therefore examined the effect of verapamil against total $[Ca^{++}]_i$ signal (ie the response measured when $[Ca^{++}]_o$ = 1 mM) and against $[Ca^{++}]_i$ that arises only from mobilization (ie. in the presence of EGTA) induced by a submaximal concentration of PAF.

The concentration response curves for inhibition of total $[Ca^{++}]_i$ and $[Ca^{++}]_i$ due to mobilization, by verapamil, were virtually superimposable (fig. 31).

With Quin2-labelled platelets suspended in the normal buffer but with no added Ca++ and in the absence of EGTA, to facilitate the manipulation of $[Ca^{++}]_o$, PAF evoked an elevation in $[Ca^{++}]_i$ which rapidly reached a peak then returned to resting levels. If $[Ca^{++}]_o$ was subsequently readjusted to 1 mM then a second elevation in $[Ca^{++}]_i$ was observed (see fig. 19). As discussed in section 7.2. the initial response was attributed to mobilization of intracellular Ca++ and the latter to influx of extracellular Ca++.

This system appeared to offer a mechanism whereby the influx component could be isolated from the component due to mobilization and hence presented the opportunity to examine the effect of verapamil on each component individually.

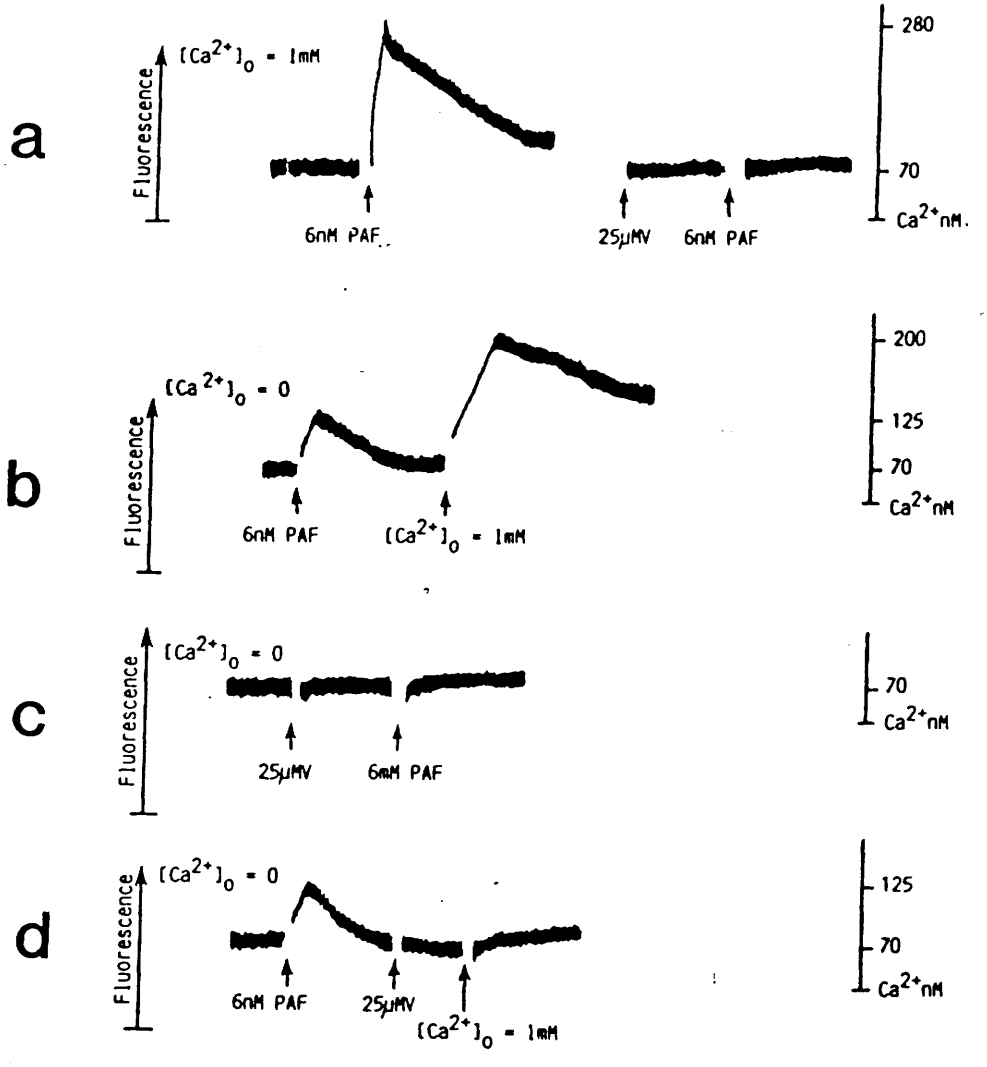

Figure 32. The effect of verapamil on PAF-induced Ca++ influx and mobilization.
Experiments were performed essentially as described for previous figs (figure 19)
(a) The normal fluorescence response to a submaximal concentration of PAF (left) and right the effect of 25μM verapamil on [Ca++] i.
(b) The change in [Ca++] i induced by PAF when [Ca++] o = 0mM (mobilization) and the effect on [Ca++] i of restoring [Ca++] o to 1mM after activation (influx).
(c) The effect of verapamil on PAF-induced Ca++ mobilization.
(d) The effect of verapamil on PAF-induced Ca++ influx.
Results are representative of at least 3 other experiments using platelets from different donors.

Figure (32) demonstrates that verapamil inhibits both influx and mobilization.

All three classes of calcium antagonist inhibit primary aggregation induced by the agonists examined. Nicardipine exhibited the least inhibitory activity. This correlates well with the lack of binding sites for the 1,4 dihydropyridines on platelets **(Glossman, et. al., 1982)**. In addition this would also exlain the inactivity of the dihydropyridine (DHP) Ca++ agonist BAY K8644. In contrast verapamil potently and selectively inhibits aggregation induced by PAF. Diltiazem potently inhibits aggregation induced by PAF and U44069 apparently indicating that the calcium channel associated with the PAF receptor is sensitive to class II and class III calcium antagonists whereas the calcium channel associated with the U44069 receptor is sensitive to class III only. Indeed the identical inhibitory pattern observed when $[Ca^{++}]_i$ was monitored instead of aggregation would appear to support calcium channel blockade as the mechanism underlying the inhibitory action of these drugs.

However verapamil and diltiazem abolish $[Ca^{++}]_i$ completely. As initial studies established that $[Ca^{++}]_i$ was composed of an influx of extracellular calcium (80 %) and mobilization of intracellular calcium (20 %), the predicted result would be a maximum inhibition of $[Ca^{++}]_i$ of 80 %.

One of two conclusions may be derived from this data.

a) As verapamil and diltiazem appear to block both influx and mobilization they are not acting purely by channel blockade.

b) If both drugs are acting by blockade of ROC's then is $[Ca^{++}]_i$ recorded in the presence of 3 mM EGTA truely mobilization of intracellular calcium or could it still represent influx of $[Ca^{++}]_o$.

The effective buffering range of EGTA for ionic calcium occurs in the low micromolar concentration range and becomes progressively less effective towards nanomolar concentrations. In addition the inaccuracy of pH meters (used to pH buffers) and calcium contamination from buffer reagents makes an accurate assessment of the free $[Ca^{++}]_o$ in the presence of EGTA particularly difficult.

Consider three possible free $[Ca^{++}]_o$ concentrations in the presence of EGTA.

a. $[Ca^{++}]_o$ = 10 nM

With a membrane potential (Vm) of - 60 mV **(MacIntyre & Rink, 1982)** and assuming a resting $[Ca^{++}]_i$ of 100 nM. The calcium equilibrium potential (Eca)

$$Eca = \frac{58 \log 10}{100}$$

$$= - 58 \text{ mV}.$$

according to the Nernst equation. Since Vm = Eca the electrical

potential driving inward calcium current would balance the outward movement of calcium down its concentration gradient. Hence under these conditions no net movement of calcium would occur, consequently $[Ca^{++}]_i$ could only be due to mobilization of intracellular calcium.

b. If $[Ca^{++}]_o$ = 50 nM

then $E_{Ca} = \frac{58 \log 50}{100}$

$= -17$ mV.

Accordingly, since Vm > Eca the electrical potential driving inward calcium current would be larger (- 43 mV) than the concentration gradient opposing it. Consequently, in this situation, the net movement of calcium would be from the extracellular environment to the cytosol. $[Ca^{++}]_i$ recorded in this environment could therefore originate from an influx of extracellular calcium rather than mobilization of intracellular calcium.

Under these conditions the cytosolic calcium concentration would have to elevated to around 500 nM before Eca = - 58 mV and therefore balance Vm. Hence in theory an extracellular Ca++ concentration of 50 nM could elevate the cytosolic calcium concentration to 500 nM.

Although $[Ca^{++}]o$ (in the presence of EGTA) cannot be measured accurately a computer programme (compiled by **Dr. D, Miller; Dept. of Physiology, university of Glasgow**) which, knowing the calcium binding constants of the buffer reagents and which accounts for the error due to pH and contamination, predicts that the free calcium concentration of this system would be 0.6nM. Assuming this value to be an accurate reflection of the real situation then the elevation in $[Ca^{++}]i$ recorded in the presence of EGTA could not be due to influx of extracellular calcium.

c. With $[Ca^{++}]o = 0.6$ nM

$$Eca = \frac{58 \log 0.6}{100}$$

$= -128$ mV, $Vm < Eca$ hence an electrical potential of (+ 66 mV) would augment the movement of calcium down its concentration gradient. Therefore a net efflux would exist, and $[Ca^{++}]i$ recorded in this environment would be smaller than than the true value.

Clearly the exact knowledge of the free calcium concentration would be required to achieve an accurate and true interpretation of the data. From the predicted free calcium concentration it would appear that $[Ca^{++}]i$, in the presence of EGTA , is a genuine mobilization of intracellular calcium, although its true value may be greater than that recorded. It follows therefore that the mechanism of action of verapamil

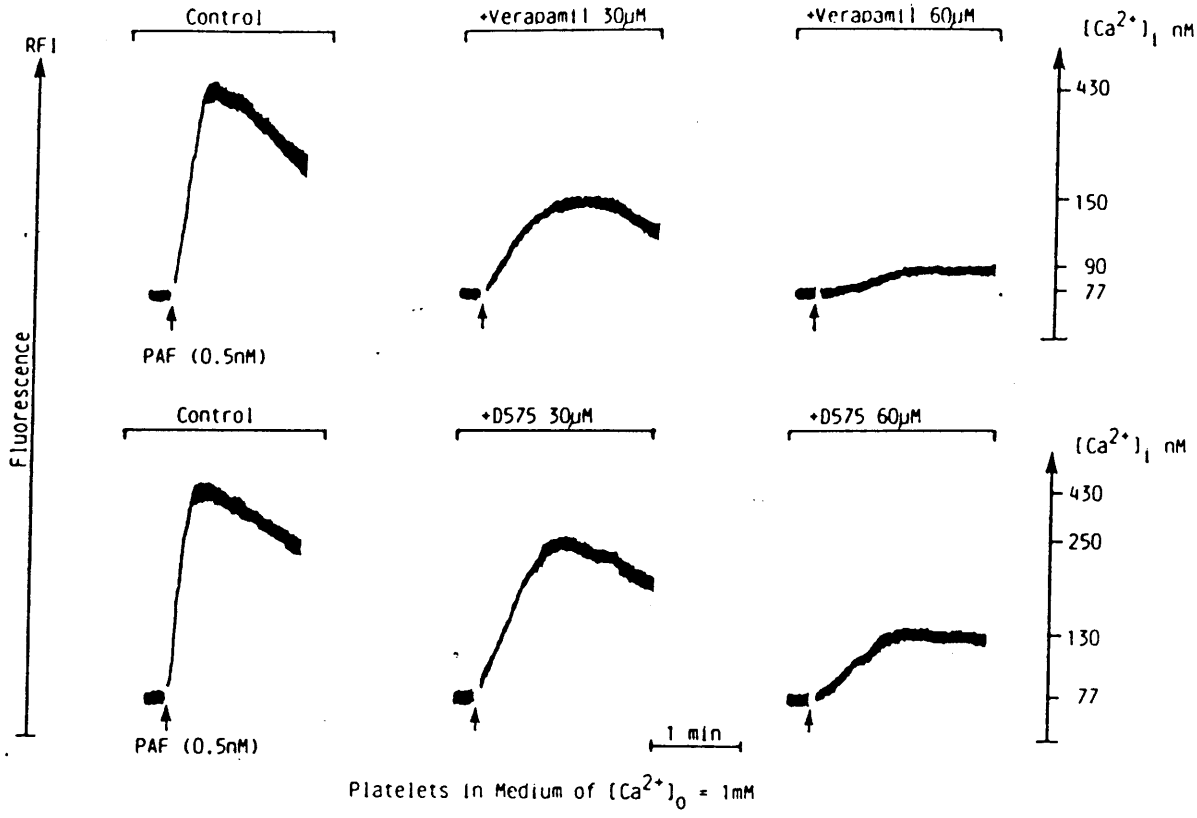

Figure 33. The effect of D 575 on PAF-induced [Ca++] i and primary aggregation. Experiments were performed as described for figure 30 Results are from single experiments and are representative of 4 similar experiments.

(and diltiazem) cannot be due purely to calcium channel blockade. Furthermore if $[Ca^{++}]_i$ recorded in the presence of EGTA is smaller than the true value, because of a net loss of calcium from the cytosol to the external environment, calcium channel blockade, which abolishes ion movements in both directions across the plasma membrane **(Tsien 1984)**, should increase $[Ca^{++}]_i$ rather than inhibit.

Finally as the site of action of the phenylalkylamines, as calcium channel blockers, is allegedly at the inner mouth of the calcium channel, the quaternary derivative of verapamil (D575), which is only effective when injected into the cell **(Glossman, et. al., 1982)** would therefore provide a useful tool to distinguish between calcium channel blockade and non-calcium channel blocking effects. Inhibition obtained with this analogue (added to the suspension medium) could not be due to channel blockade. Fig. 34 shows that D575 inhibited changes in cytosolic free calcium induced by PAF in a manner similar to verapamil.

8.5. COULD OTHER PHARMACOLOGICAL ACTIONS OF VERAPAMIL ACCOUNT FOR THE SELECTIVE INHIBITION OF PAF-INDUCED PLATELET ACTIVATION?

The phenylalkylamines are known to act as alpha-adrenoceptor antagonists **(Fairhurst et. al., 1980)**, as sodium channel blockers **(Frelin et. al., 1982)**, as inhibitors of cAMP phosphodiesterase **(Epstein et. al., 1982)**, and as local anaesthetics **(Hay & Wadsworth, 1982)**. Could such actions

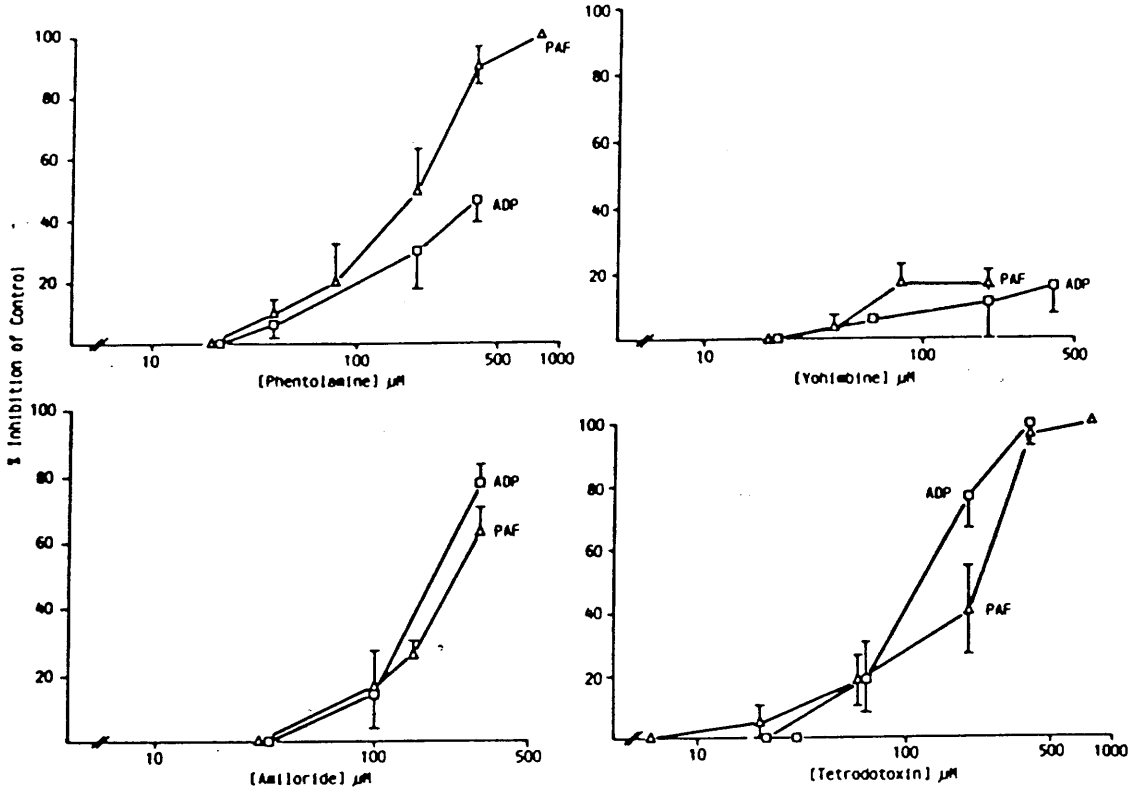

Figure 34. Effects of sodium channel blockers and alpha-adrenoceptor antagonists on primary aggregation induced by submaximal concentrations of PAF and ADP. Experiments were performed essentially as described for CCBs fig. 30. Results are the mean values ± SE from 3 - 4 experiments using platelets from different donors.

	IC_{50} value (μM) versus agonist:			
Drug	PAF	(n)	ADP	(n)
Phentolamine	190 ± 50	(4)	> 200	(3)
Yohimbine	> 200	(3)	> 200	(3)
Tetrodotoxin	280 ± 60	(4)	115 ± 25	(3)
Amiloride	220 ± 40	(3)	185 ± 25	(3)

Table 4. The effect of sodium channel blockers and alpha-adrenoceptor antagonists on PAF- and ADP-induced platelet aggregation. Experiments were performed essentially as described for the calcium antagonists in table 3. The results are the mean values ± S.E. from the observations (n) indicated.

account for the selective inhibition of PAF-induced platelet aggregation and elevation of $[Ca^{++}]i$?

Verapamil (300uM) had no effect on platelet cAMP levels when assayed using PRP (9.4 $\pm$ 1pmol/10^8 cells compared to control 8.0 $\pm$ 0.4 pmol/ 10^8 cells; means $\pm$ SE from three experiments) indicating that phosphodiesterase inhibition cannot explain the selective inhibition of PAF-induced platelet activation. Moreover an elevation in cAMP levels leads to a non-specific inhibition of platelet reactivity. A similar argument (ie non-specific inhibition) applies to its local anaesthetic activity.

To exclude the possibility that selectivity may be due to antagonism of alpha-adrenoceptors or sodium channel blockade the inhibitory effect of verapamil was compared to that evoked by specific alpha adrenoceptor antagonists and sodium channel blockers.

Concentration response curves for inhibition by tetrodotoxin and amiloride (sodium channel blockers) and phentolamine and yohimbine (alpha-adrenoceptor antagonists), of aggregation induced by PAF and ADP were constructed (fig. 34) and from these a table of IC_{50} values estimated (table 4).

Yohimbine had little effect on aggregation induced by ADP or PAF, producing less than 20 % inhibition even at the highest concentrations. Amiloride, phentolamine and tetrodotoxin inhibited PAF- and ADP-induced aggregation in a concentration dependent manner but only at high concentrations. Hence these results exclude alpha adrenoceptor antagonism or sodium channel blockade as the mechanism of action of verapamil on PAF-induced

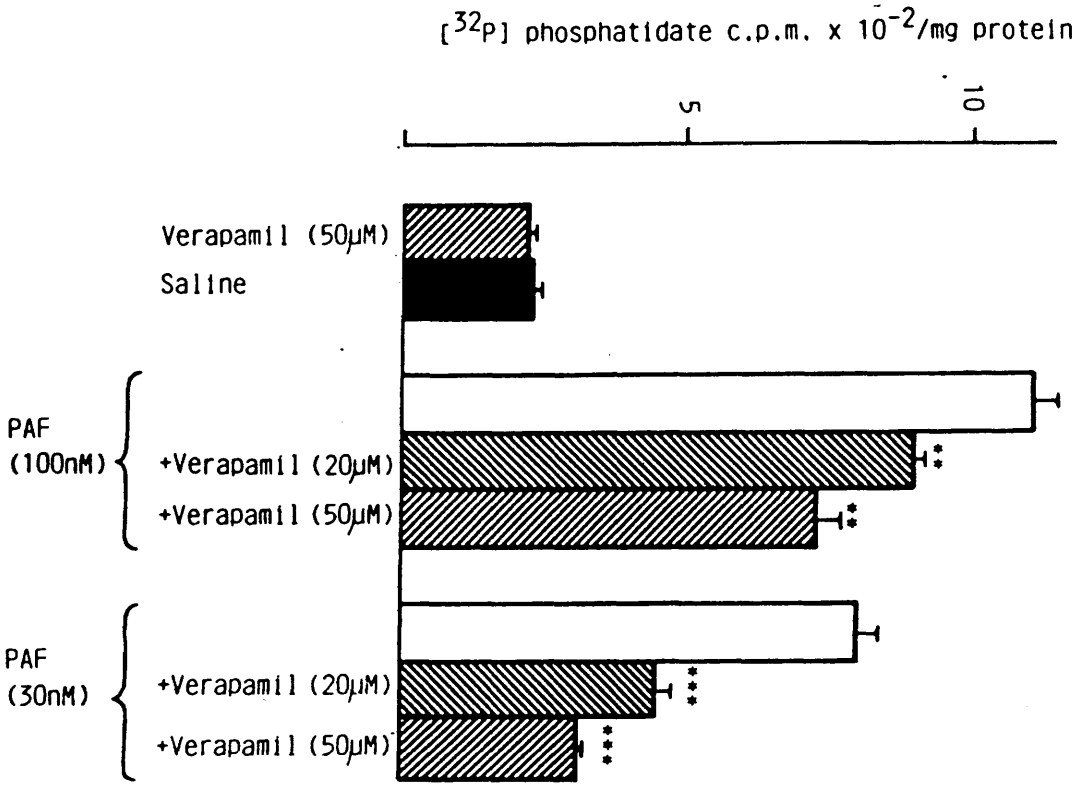

Figure 35. The effect of verapamil on PAF-induced Phosphatidic acid formation. Verapamil at the concentrations indicated were incubated with aliquots (400 μl) of [^{32}P]-labelled platelets for 2 minutes. PAF 30 nM or 100 nM was added to initiate PI hydrolysis. The reactions were terminated 5 minutes later by transferring the platelet sample into 2 ml of chloroform/methanol/HCL and platelet lipids extracted and separated as described in experimental procedure. Results are the mean values from 3 experiments conducted in duplicate.

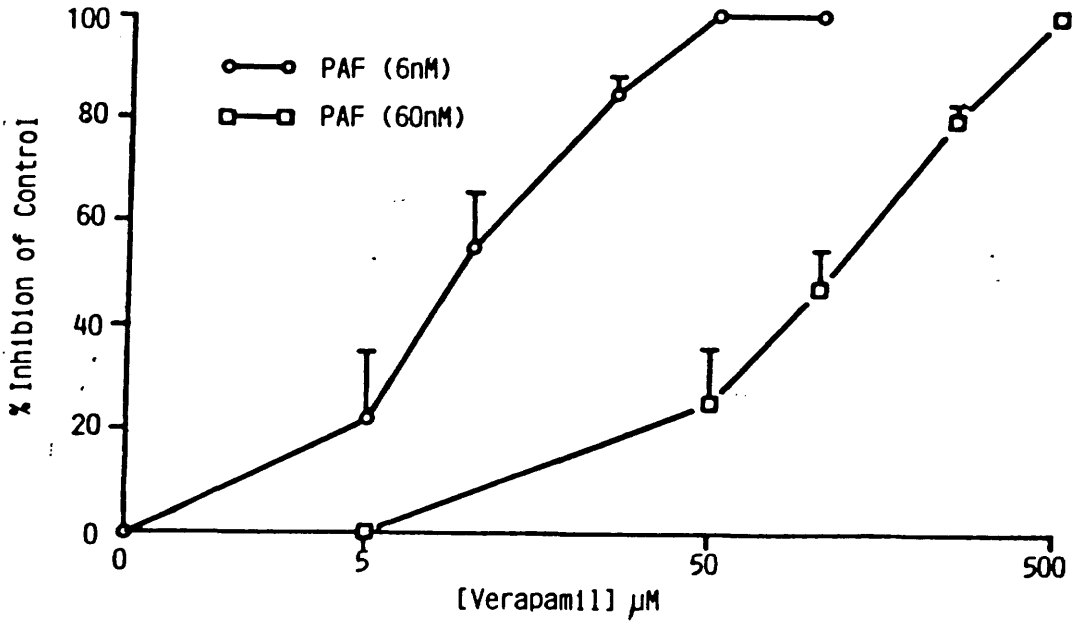

Figure 36. The effect of agonist concentration on inhibition, by verapamil, of PAF-induced elevations of [Ca++] i. Verapamil, at the concentrations indicated on the abscissa, was preincubated with quin2 loaded platelets for 2minutes. PAF (6 nM or 60 nm) was added to initiate platelet activation. The changes in fluorescence was recorded from which the changes in [Ca++] i were calculated. The results are the mean values ± S.E. from 3 experiments and are expressed as percentage inhibition of the control response.

platelet activation.

8.6. THE EFFECT OF VERAPAMIL ON INOSITOL PHOSPHOLIPID HYDROLYSIS.

The hydrolysis of the inositol phospholipids has been suggested to be the mechanism by which agonists elicit an elevation in cytocolic free calcium. As that includes both influx and mobilization (see section 4.3.) then its inhibition (inhibition of inositol phospholipid hydrolysis) by verapamil would account for the observation that this agent abolished both influx and mobilization. Figure 35 shows that, like aggregation and $[Ca^{++}]_i$ induced by PAF, $[^{32}P]$- PA formation (a monitor for PI hydrolysis) is also inhibited by verapamil.

Against 100 nM PAF, 20 μM and 50 μM verapamil inhibited the agonist response by 38 ± 13 % and 55 ± 10 % (means ± SD from three experiments) respectively. Against 30 nM PAF 20 μM and 50 μM verapamil inhibited the agonist response by 46 ± 21 % and 60 ± 30 % (means ± SD from three experiments) respectivly.

As all agonist which induce an elevation in cytosolic free calcium induce inositol phospholipid hydrolysis its inhibition by verapamil would evoke a non-specific inhibitory response. Therefore the action of verapamil must occur at a point preceeding this event. It was noted (fig. 36) that the concentration response curve for inhibition of cytosolic free calcium could be displaced to the right by increasing the concentration of PAF, indicating a competitive interaction

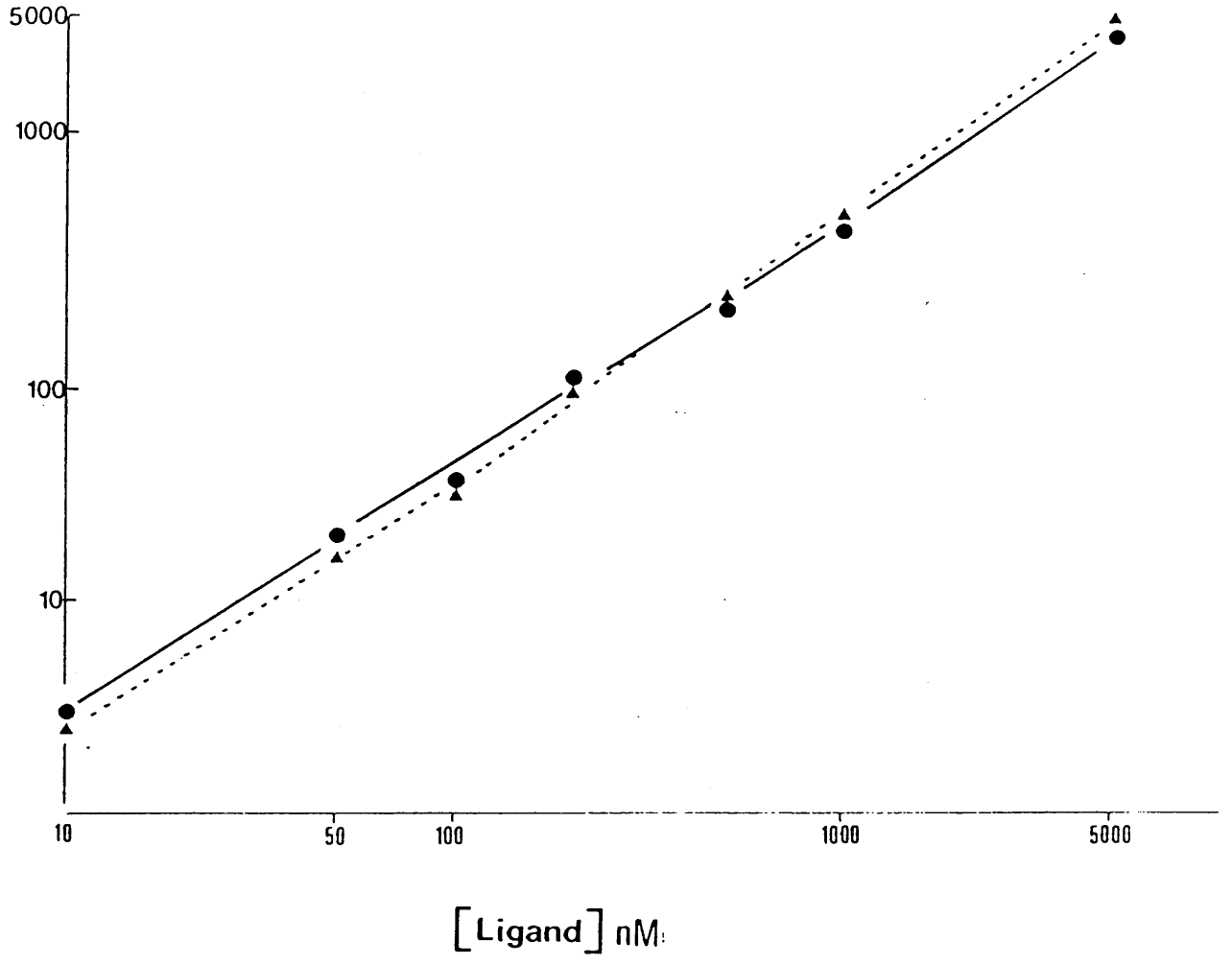

Figure 37. $[^3H]$- PAF binding to rabbit platelets. Aliquotes of plasma free suspensions of rabbit platelets were incubated with 1-O-alkyl-1'-2' $[^3H]$-2-acetyl glycerophosphocholine (New England Nuclear) in the presence and absence of excess unlabelled PAF. Icubations were terminated by rapid centrifugation. After the supernatant was aspirated the platelet pellet was digested with hyamine hydroxide and the radioactivity in each aliquote measured by liquid scintilation counting. The results show the concentration of free ligand bound (ordinate) in the presence (●——●) and absence (▲-----▲) of excess PAF versus the concentration of ligand added (abscissa). The results from one experiment were typical of 5 similar experiments.

between PAF and verapa il. Hence the posibility exists that verapamil may be acting as a PAF receptor antagonist. Several attempts to show a specific binding site for PAF were unsuccessful in that no high affinity (saturable) binding site was detected. A typical binding curve is shown in figure 37. However other workers : **Valone, et. al. (1984); Chessney, et. al. (1983) and Wade, et. al. (1984)** have been able to demonstrate a high affinity binding site of Kd = 37 nM; 0.7 nM and 0.18 nM respectively. The latter group have reported that verapamil displaced $[^{3}H]$-PAF binding with an IC_{50} of 30 nM. The reason why my attempts to demonstrate specific binding sites for PAF were unsuccessful is unclear. However, more recently it has been shown that the radioligand used in this study for some unknown reason fails to bind to PAF receptors **(Kloprogge & Akkerman 1983)**

Hence the data presented in this section suggests that the inhibition of PAF-induced platelet activation by the phenylalkylamines (and diltiazem) is not mediated by blockade of a specific calcium channel. Rather receptor antagonism may be the mechanism underlying the specific inhibition of PAF-induced events

9. PHARMACOLOGICAL MANIPULATION OF THE CYTOSOLIC FREE CALCIUM CONCENTRATION: CYCLIC NUCLEOTIDES

In platelets, adenylate cyclase stimulants and exogenous cAMP analogues inhibit aggregation and the release reaction induced by all known agonists **(Salzman et. al., 1972; Haslam, 1975)**. Similarly, aggregation and secretion induced by collagen, adrenaline, ADP, arachidonic acid, A23187 and thrombin are inhibited by guanylate cyclase stimulants and by exogenous cGMP analogues **(Nishikawa et. al., 1982)**. It would seem likely, therefore, that these nucleotides act by inhibiting a mechanism common to all excitatory agonists. As several studies have shown the ability of cAMP to enhance Ca++ sequestration, not only in platelets, but also in microsomal fractions of various smooth muscle preparations **(Anderson & Nilsson, 1972)**, it has been proposed that the inhibitory action of cAMP may be due to reducing the availability of $[Ca^{++}]_i$. In addition, besides promoting Ca++ sequestration, cAMP also may impair the processes (mobilization and influx) that promote elevation of $[Ca^{++}]_i$

In vascular smooth muscle **Schultz et. al. (1977)** have proposed that the relaxation associated with elevated cGMP levels in this tissue is due to the redistribution of $[Ca^{++}]_i$. In support of this view **Zsotér et. al. (1977)** have shown that cGMP enhances $^{45}Ca^{++}$ efflux in certain types of smooth muscle.

Cyclic AMP- and cGMP-dependent protein kinases have been shown to phosphorylate the same proteins, although to varying extents, in platelets **(Haslam et. al., 1980)** and in other tissues: for example rabbit skeletal muscle glycogen synthetase and phosphorylase b kinase, and rat pyruvate kinase and

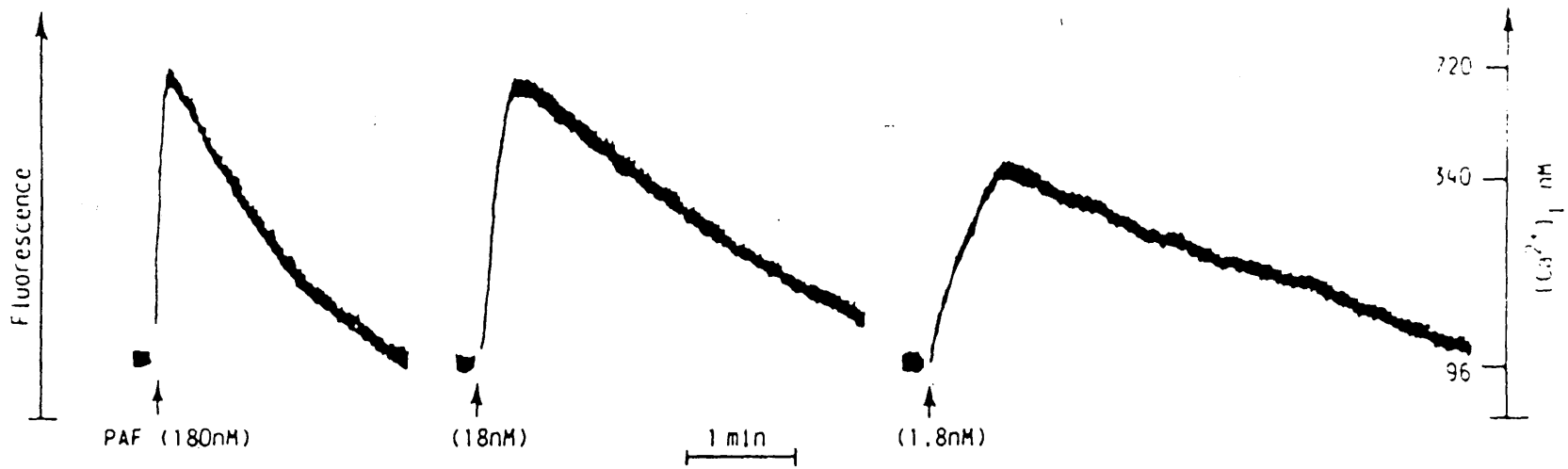

Figure 38. The effect of agonist concentration on the rate of restoration of [Ca++] i following the agonist-induced elevation. PAF at the concentrations indicated was added to quin2 labelled platelets and the fluorescence changes recorded as described before.

fructose 1,6, diphosphatase **(Lincoln & Corbin, 1977)**. In most cases cAMP kinase appears to be the more efficient catalyst.

Although circumstantial, the evidence tentatively suggests that cAMP and cGMP may exert their inhibitory action by the same mechanism, possibly suppression of $[Ca^{++}]_i$ availability. Hence in this study I examined the effects of cAMP and cGMP on platelet $[Ca^{++}]_i$ and attempted to correlate the inhibitory action of various agents that mimic or elevate cAMP or cGMP on platelet functional response (aggregation) and on $[Ca^{++}]_i$ induced by PAF.

Finally, it was noted that the rate of $[Ca^{++}]_i$ restoration following agonist stimulation was directly proportional to agonist concentration (fig. 38). Such an observation could be explained by the phenomenon of homologous receptor desensitization or perhaps by the generation of some endogenous mediator that promotes calcium sequestration and/or calcium extrusion.

Potential endogenous regulators may include cAMP and cGMP. Formation of cAMP by platelets in response to stimulatory agonists has not been reported, therefore it is unlikely that this nucleotide would subserve such a regulatory mechanism. However in certain smooth muscle systems, a rise in the intracellular concentration of cGMP has been reported following the initial contractile event. Since this nucleotide promotes relaxation, allegedly through redistribution of $[Ca^{++}]_i$, it has

been proposed that cGMP might function in a negative feedback mechanism limiting cellular response. **(Schultz, 1977)**. That a similar mechanism might operate in platelets has been suggested by **Haslam (1980)**. In support of such a role, though not a universal observation, some workers have reported that an elevation in intraplatelet cGMP levels follows stimulation **(Haslam & McClenaghan, 1974)**. Moreover platelet stimulation is also followed by an enhanced efflux of $^{45}Ca^{++}$ **(Massini & Luscher, 1974)**. Guanylate cyclase stimulants include unsaturated fatty acid peroxides **(Hidaka & Asano, 1977)**, which in platelets, can be formed from arachidonic acid metabolism following stimulation.

Therefore it is conceivable that following platelet activation a late rise in the intracellular level of cGMP is responsible for restoring the cell to its former unactivated state. A possible sequence of events, following receptor occupation, could be:-

(1) an agonist mediated elevation in $[Ca^{++}]_i$.

(2) platelet functional responses.

(3) mobilization of arachidonic acid and its metabolism via cyclooxygenase (CO) and lipoxygenase (LO).

The metabolism of arachidonic acid via LO is known to lag behind that of CO **(Samuelsson, et. al., 1976)**

(4) the formation of hydroperoxy-metabolites of arachidonic acid, which simulate guanylate cyclase, could represent one mechanism underlying a delayed rise in cGMP.

I therefore examined the effect of cGMP and cAMP on $[Ca^{++}]_i$ restoration and measured the platelet concentrations of these

nucleotides after stimulation by PAF, thrombin and U44069 to determine whether they could form part of a negative feedback mechanism in these cells.

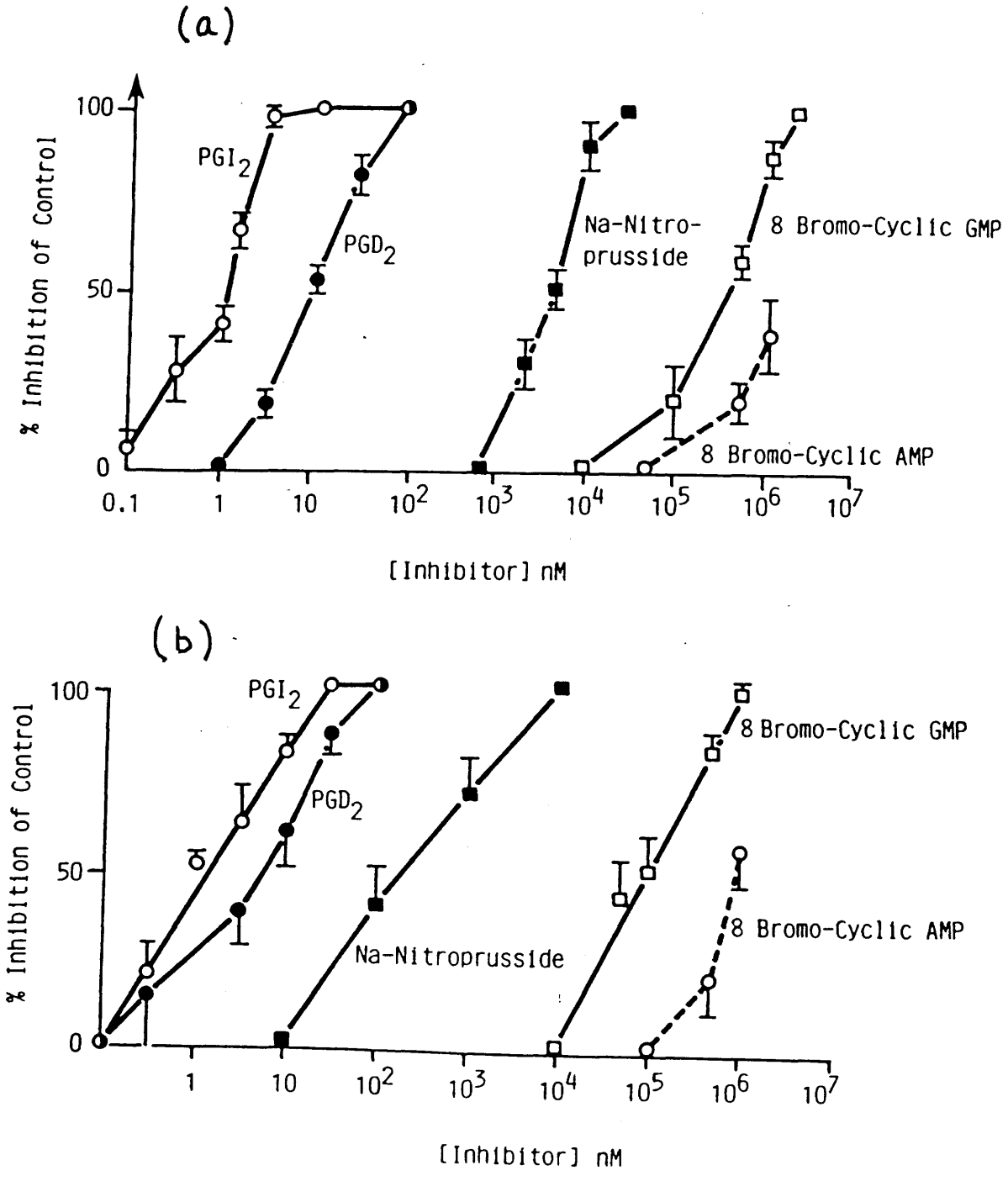

Figure 39. The effect of PGI_2, PGD_2, NaNP, 8Br cAMP and 8Br cGMP on a). primary aggregation induced by submaximum concentrations of PAF and b). elevations in [Ca++] i induced by submaximum concentrations of PAF. PGI_2, PGD_2 or NaNP was preincubated for 2 minutes and 8Br cAMP and 8Br cGMP for 5 minutes with PRP, for aggregation studies, or in plasma free medium with quin2 loaded, for [Ca++] i studies. PAF was added to initiate aggregation or changes in [Ca++] i. and the responses recorded as described before. The results are the mean values ± S.E. from 4 experiments and are expressed as % inhibition of the control response.

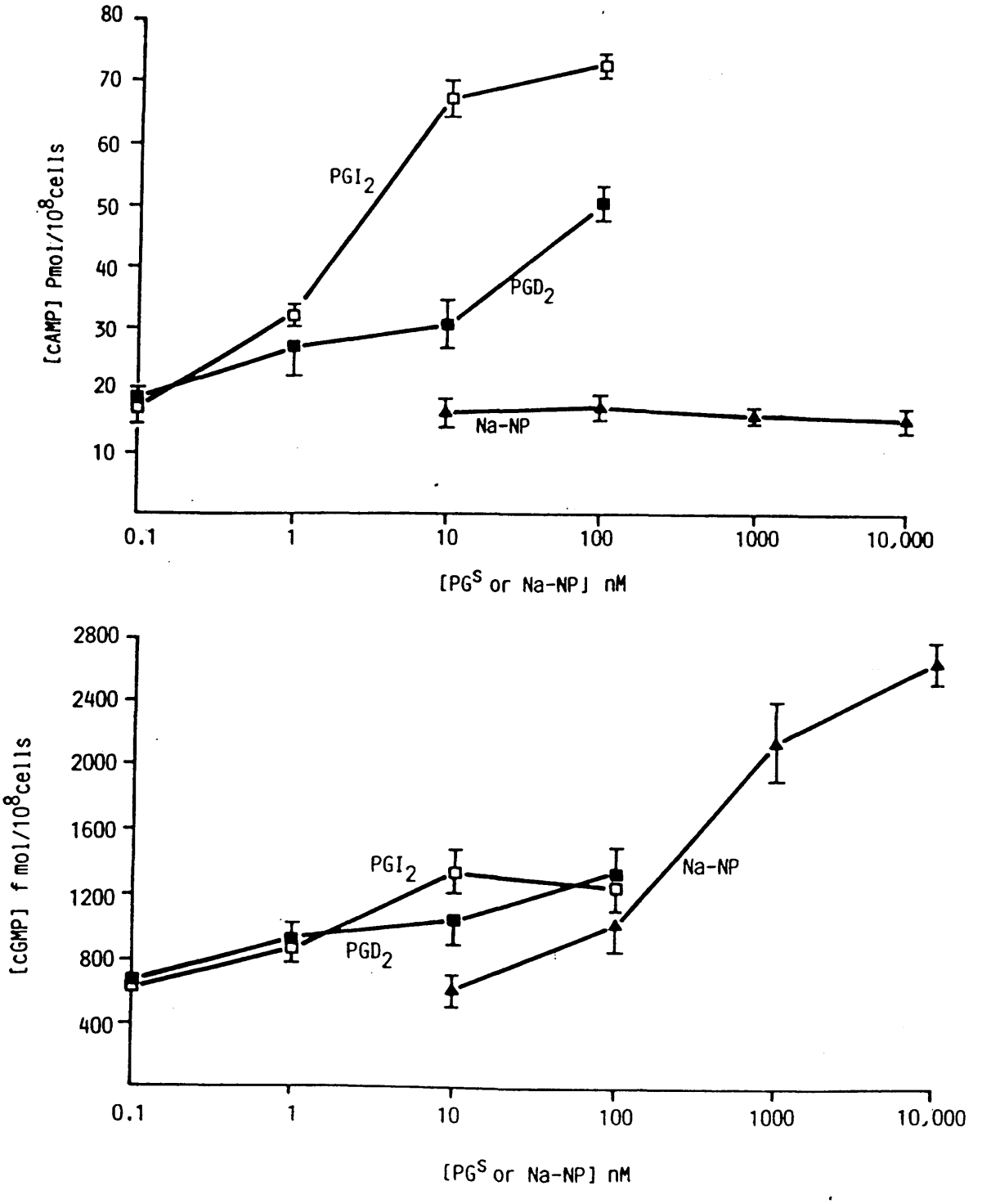

Figure 40. The effect of PGI_2, PGD_2 and NaNP on total platelet cAMP and cGMP concentration. PGI_2, PGD_2 and NaNP were added to a plasma free suspension of platelets at 37°C for 2 minutes (unstirred). Reactions were terminated and nucleotides extracted by the addition of ethanol (1 vol.). The cyclic nucleotide content in the organic phase was then determined by radioimmunoassay as described in section 5.6. Results are the mean values ± S.E from 4 - 8 experiments.

9.1. INHIBITION OF PLATELET FUNCTIONAL RESPONSE BY CYCLIC NUCLEOTIDES: CORRELATION WITH INHIBITION OF $[Ca^{++}]_i$.

The inhibitory effect of PGI_2, PGD_2 (which stimulate adenylate cyclase), sodium nitroprusside (NaNP) (which stimulates guanylate cyclase), 8-bromo-cyclic AMP and 8-bromo-cyclic GMP on submaximal primary aggregation induced by PAF is shown in figure 39 a. PGI_2 (IC_{50} = 1 ± 0.5 nM), PGD_2 (IC_{50} = 10± 0.2 nM), NaNP (IC_{50} = 4.8 ± 1 μM), 8-bromo-cGMP (IC_{50} = 350 ± 30 μM) and 8-bromo-cAMP (I_{50} > 1 mM) produced a concentration-dependent inhibition of PAF-induced aggregation.

The elevation in $[Ca^{++}]_i$, from 90 ± 3 nM resting to 250 ±50 nM induced by a submaximal concentration of PAF (80 nM) was inhibited in a concentration dependent manner (fig. 39 b) by PGI_2 (IC_{50} = 2.07 ± 0.05 nM) > PGD_2 (IC_{50} = 6.0 ± 2 nM), > NaNP (IC_{50} = 220 ± 40 nM) > 8-bromo-cGMP (IC_{50} = 140 ± 40 μM) > 8-bromo-cAMP (IC_{50} > 1mM) (means ± SE of 4 experiments).

To investigate a possible causal relationship between inhibition of $[Ca^{++}]_i$, and platelet cAMP or cGMP, platelet concentrations of these nucleotides were measured over the active concentration ranges of PGI_2, PGD_2 and NaNP (fig. 40). In the presence of a phosphodiesterase inhibitor (IBMX, 100uM) resting platelet cAMP and cGMP were 12 ± 1 pmol and 315 ± 20 fmol/10^8 cells respectively (means ± SE of 4 - 8 experiments). PGI_2 and PGD_2 evoked a concentration-dependent elevation in both cAMP and cGMP.

PGI_2 (1 nM) and PGD_2 (10 nM) (the nearest concentration to the IC_{50} values measured for inhibition of $[Ca^{++}]i$) produced an elevation in cAMP of $\simeq$ 2.5- and $\simeq$ 3.5- fold respectively. In contrast NaNP (100 nM) stimulated the formation of cGMP only ($\simeq$ 3 fold).

This study has shown that agents which elevate cAMP or cGMP inhibit aggregation and $[Ca^{++}]i$ induced by PAF. Furthermore the rank order of potency of agents that inhibit aggregation was identical to that for inhibition of $[Ca^{++}]i$ suggesting that the suppression of $[Ca^{++}]i$ availability could be the mechanism underlying the action of these nucleotides.

The potency of NaNP and 8Br cGMP as inhibitors of $[Ca^{++}]i$ clearly differs from their potencies for inhibition of aggregation. Platelet aggregation was monitored using PRP whereas $[Ca^{++}]i$ was measured using washed platelets. Hence, although unlikely binding to plasma proteins may account for the observed difference in potency of NaNP and 8Br cGMP in the two systems.

Interestingly PGI_2 and PGD_2 elevate both cAMP and cGMP. Similar findings were observed by **Bruckdorfer et. al. (1984)**. The findings with NaNP which inhibits elevation of $[Ca^{++}]i$ at concentrations that promote synthesis of cGMP but not cAMP, and the effects of 8Br cGMP clearly indicate that cGMP can regulate agonist-induced elevation of $[Ca^{++}]i$. Could the elevation in cGMP explain the inhibitory action of PGI_2 or PGD_2, PGI_2 (10 nM) and PGD_2 (100 nM) virtually abolish elevation of $[Ca^{++}]i$, yet the rise in cGMP is similar

to that produced by 100nM NaNP. The inhibition produced by 100nM NaNP (ie. increased cGMP only) is less than 50 % of the control response. Clearly, while cGMP may contribute to the inhibition , the major effect must be due primarily to cAMP.

It could be argued that if cAMP and cGMP were metabolized by the same phosphodiesterase then incomplete inhibition of this enzyme could account for the elevation in cGMP produced by PGI_2 and PGD_2. For example if the metabolizing capacity of the phosphodiesterase were reduced (by IBMX) to a point where the elevated level of cAMP (induced by PGI_2 and PGD_2) was competing with cGMP (formed by basal turnover) then an elevation in the latter nucleotide would occur. Assuming competition between these two nucleotides for a limited metabolic capacity of the phosphodiesterase then changes in cGMP should occur in parallel with changes in cAMP. Clearly the results show this not to be so, moreover a specific cGMP phosphodiesterase has been identified in platelets **(Asano & Hidaka, 1977).**

A similar argument may be applied to the suggestion that high concentrations of cAMP may cross react with the cGMP antibody. Again if this were the situation the apparent elevation in cGMP would have to parallel any elevation in cAMP.

From these results it must be concluded that the elevation in cGMP induced by PGI_2 and PGD_2 is independent of their ability to stimulate adenylate cyclase.

One might expect that agents which inhibit agonist-induced elevations in $[Ca^{++}]_i$ also promote sequestration or extrusion of Ca^{++}. Again the potential endogenous regulators would include

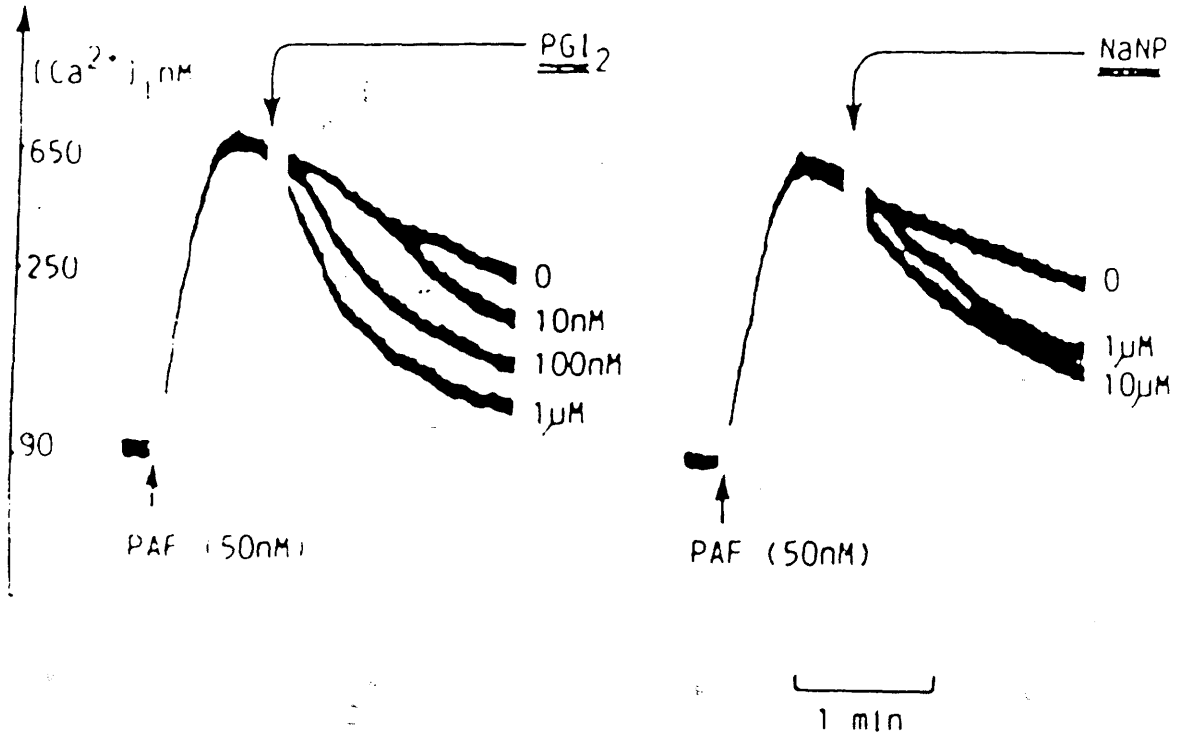

Figure 41. The effect of adenylate or guanylate cyclase stimulants on the rate of restoration of [Ca++] i following an agonist-induced elevation. PGI_2 or NaNP at the concentrations indicated were added at the peak of the elevation of [Ca++] i induced by PAF. The results are from a single observartion but are typical of at least 4 observations using platelets from different donors.

cAMP and cGMP. Indeed addition of adenylate cyclase or guanylate cyclase stimulants accelerate the restoration of agonist-induced changes in $[Ca^{++}]_i$ (fig 41). To assess the potential role of cAMP and cGMP, as mediators of $[Ca^{++}]_i$ restoration, the levels of these nucleotides were monitored, in the presence of a phosphodiesterase inhibitor, following stimulation by a supramaximal concentration of PAF (500nM). When compared to controls (unstimulated cells) no significant elevation in cAMP or cGMP was detected for up to 5 min. of agonist addition; by which time $[Ca^{++}]_i$ returned to normal values. Similar results were obtained with thrombin and U44069 (data not shown).

9.2. IS THE INHIBITORY ACTION OF cAMP AND cGMP MEDIATED BY THEIR ABILITY TO SUPPRESS THE AVAILABILITY OF CYTOSOLIC FREE CALCIUM ?

The results of this study show that pretreating the cells with adenylate or guanylate cyclase stimulants can prevent changes in $[Ca^{++}]_i$.

Does the available evidence suggest that these nucleotides mediate their inhibitory effect by suppressing the availability of $[Ca^{++}]_i$.

In theory two mechanisms exist by which these agents might suppress agonist-induced elevations in $[Ca^{++}]_i$. They could act by inhibiting the mechanism responsible for increasing the cytosolic calcium concentration (ie. the mechanism that regulates Ca++ influx and/or mobilization from intracellular

sites). As inositol phospholipid metabolism is currently believed to be the mechanism responsible for Ca++ "gating", then its inhibition would clearly be an essential target for these nucleotides. Indeed both adenylate and guanylate cyclase stimulants have been shown to inhibit agonist-induced PI hydrolysis **(Takai et al. 1981; MacIntyre et al 1985).**

In permeabilized platelets **Knight & Scrutton (1984)** have shown that $[^{14}C]$-5HT can be released in a concentration dependent manner by increasing the concentration of extracellular calcium (in this system small ions, including Ca++, in the buffer medium can equilibrate with the cytosol). Both thrombin and OAG displace the concentration response curve for Ca++ to the left (presumably by activating protein kinase C - the Ca++ - independent mechanism for platelet activation: section . Cyclic AMP was able to suppress the effects of thrombin but had little effect on the enhancement evoked by OAG. As OAG mimics diacylglycerol, the endogenous activator of PKC formed by agonist-induced hydrolysis of PI, these results indicate that the target for cAMP must preceed activation of PKC but must prevent agonist-induced formation of DAG. DAG formation results from the action of a PLC enzyme on the inositol phospholipids therefore the most likely target for cAMP would be the inhibition of this enzyme.

In permeabilized cells cGMP enhances the effect of thrombin. This is in contradiction to its effect in intact cells. Haslam has also noted this anomaly, but suggests that this is due to the metabolic conversion of cGMP to GTP or other guanine nucleotides, which enhance agonist-induced 5HT release

(Haslam & Davidson, 1984) Clearly the non-metabolizable 8 Br-cGMP should resolve this problem.

It is possible therefore, that cAMP and cGMP, via their respective kinases, suppresses the agonist-induced elevation in cytosolic free calcium by preventing agonist-induced PI hydrolysis. However, as both nucleotides suppress the cellular response to adrenaline; an agonist which does not evoke PI hydrolysis or changes in cytosolic calcium, then these nucleotides must also be capable of evoking an inhibitory response which does not involve Ca++ metabolism.

A second mechanism by which these nucleotides could prevent agonist-induced elevations in cytosolic free calcium could be to stimulate calcium sequestration and extrusion, such that, even with continued influx or mobilization the cytosolic Ca++ concentration remains low. In this study both adenylate and guanylate cyclase stimulants accelerate the restoration of agonist-induced elevations in $[Ca^{++}]_i$. In early studies cAMP was shown to accelerate the ATP-dependent uptake of Ca++ by platelet membrane fractions **(Kaser-Glanzman et al., 1971).**

Cyclic AMP phosphorylates four proteins in platelets with molecular weights of 50, 36, 24 & 22 Kdaltons (Kd). The 24 Kd peptide is associated with a membrane fraction, capable of Ca++ uptake **(Haslam, 1980).** Recent studies have isolated the platelet Ca++ "pump". It is interesting to note from these studies that, even though stimulatory agonists are not reported to elevate the cytosolic concentration of cAMP, the principle regulator of this "pump" is a cAMP dependent-kinase. The intensity of phosphorylation of the 24 Kd peptide parallels the

rate of Ca++ uptake **(Levy-Toledano et al., 1985)**. One would expect that if this "pump" is involved in Ca++ homeostasis then its principle regulator, cAMP, would also be involved in Ca++ sequestration.

Guanylate cyclase stimulants selectively phosphorylate a 49 Kd peptide and also the 50 Kd peptide to the same extent as cAMP. However the role of these proteins is unknown. Guanylate cyclase stimulants also phosphorylate the 24 Kd peptide but to a much smaller extent than cAMP. Hence cAMP appears to be capable of promoting Ca++ "pump" activity in platelets, though a similar role for cGMP is less clear.

Finally, the Ca++-induced $[^{14}C]$5HT release in permeabilized platelets is unaffected by cAMP. In this system Ca++ extrusion/sequestration would not be expected to alter the cytosolic Ca++ concentration as both external and internal Ca++ are in equilibrium. This however suggests that normally, in intact cells where cAMP inhibits 5HT release, extrusion/sequestration of Ca++ must play an important role in the inhibitory response evoked by this nucleotide.

GENERAL DISCUSSION.

PAF AND LPA: putative lipid mediators of platelet activation.

Both PAF and LPA are produced by activated platelets. Exogenous PAF and LPA can induce platelet activation though dense body release evoked by these lipids is mediated by the endoperoxides or thromboxane A_2. PAF- or LPA-induced release of alpha granule or lysosomal constituents has not been examined but may also require the formation of these icosanoids as intermediaries.

Although PAF is generated by activated platelets its contribution to agonist-induced platelet activation (ie. as an endogenous mediator propagating platelet activation) is unclear. PAF formed during platelet activation was originally hypothesised as mediating the ADP/TXA_2 independent pathway of platelet activation induced by thrombin. To date a specific receptor antagonist of PAF has not been available. uch an agent would clearly be a valuable tool to determine the importance of PAF in platelet reactivity. The results of the present study suggest that analogs of the phenylalkylamines may fulfil such a role.

One approach which has been used to demonstrate the importance of endogenous mediators in an agonist-induced response has been to make use of the phenomenon of homologus desensitization - that is platelets pre-exposure to an agonist are desensitized to subsequent challenge by the same agonist. In such a study **(Wal et al.,(1985).** reported that platelets desensitized to

PAF aggregate normally to high concentrations thrombin, in the presence of aspirin and ADP scavengers, but are less responsive to low concentrations of thrombin.

A second approach to elucidate the importance this lipid has been to inhibit the enzymes involved in the formation of PAF. These experiments have yielded essentially the same results as the desensitization experiments. That is a reduced sensitivity of the platelet to low concentrations of thrombin but no significant effect with high concentrations of thrombin **(Touqui et al., 1984).**

Therefore PAF may have some role in propagation the response to low concentrations of thrombin, as do cyclooxygenase metabolites of arachidonic acid and released ADP. From these results it would appear that PAF does not contribute significantly to mediating the ADP/TXA_2 independent platelet response induced by high concentrations of thrombin. It remains a posibility that PAF may be important in mediating the responses of other platelet agonists.

The function of PAF generated by activated platelets, if not propagation of the platelet response, is therefore unclear. In whole animal experiments PAF produces hypotension and bronchoconstriction thus platelet derived PAF may have a more important role in mediating inflammatory and allergic responses rather than as a mediator of the haemostatic response.

Although relatively high concentrations of LPA are required to induce platelet activation it has been demonstrated that under specific conditions low concentrations (2 - 20 μM) are capable

of inducing activation **(Benton et al., 1982)**. Again the role of LPA generated during platelet activation remains to be fully assessed. In a recent study **McCrea et al. (1985)** have shown that quinacrine inhibits thrombin-induced aggregation, serotonin secretion, formation of LPA, arachidonic acid release and phosphorylation of the 40/47 Kd substrate of protein kinase C. It was suggested that quinacrine inhibited both PLC and PLA_2 to produce this effect. In the same study it was found that the addition of LPA (2.5 - 22 μM) reversed the quinacrine block of thrombin-induced aggregation, protein phosphorylation and secretion of serotonin without overcoming thrombin-induced arachidonic acid release. It was concluded from these results that endogenous LPA mediates part of the response induced by thrombin. Interestingly, and in contrast to the results of the present study, LPA was able to evoke serotonin release in the absence of arachidonic acid metabolism.

Again the lack of a specific receptor antagonist for LPA severely handicaps the study of this lipid in the role of platelet activation. Like PAF, platelets pre-exposed to LPA exhibit the phenomenon of homologous desensitization. Therefore perhaps the application of this technique, in a manner similar to that used in the PAF studies, may throw some light on the role of endogenous LPA. Platelets desensitized to LPA should exhibit a reduced sensitivity to thrombin if LPA is involved in propagating the response of this agonist.

REGULATION OF PLATELET CYTOSOLIC FREE CALCIUM.

The present results show that the extent to which agonists elevate cytosolic free calcium in platelets correlates closely with the rank order of potency of those agonists, in evoking a functional response. Thus the concentration of $[Ca^{++}]_i$ would appear to be important in linking receptor occupancy to functional response. However agonists can elevate $[Ca^{++}]_i$ to similar extents without displaying the same efficacy when a functional response is measured in parallel. Suggesting that one, or more, intermediaries, other than $[Ca^{++}]_i$, are involved in linking receptor occupancy to functional response. For example PAF and thrombin can elevate $[Ca^{++}]_i$ to similar extents but thrombin is a more potent inducer of serotonin secretion than PAF. Thrombin is a more effective inducer of PI hydrolysis than PAF **(MacIntyre et al., 1985)**. The generation of diacylglycerol (a Ca++ independent intermediary, section 3 & 4) would be greater for thrombin than PAF, and this may account for the ability of thrombin to evoke a greater dense body release. The elevation of $[Ca^{++}]_i$ apparently originates principally from an influx of extracellular Ca++ presumably through "channels" in the plasma membrane, which are insensitive to changes in the transmembrane potential and the organic calcium channel blockers. Platelets are also capable of mobilizing intracellular Ca++.

The biochemical events underlying Ca++ influx or mobilization are unclear though the generation of IP3 by agonist-induced hydrolysis of PI presents a plausible mechanism by which

mobilization may be evoked.

Membrane glycoprotein complex IIb/IIIa which serves as the fibrinogen receptor (section 2) and is also the site for high affinity Ca++ binding may be involved in the Ca++ influx. Normal platelets have 45000 IIb/IIIa sites per cell. Thrombasthenic platelets have less than 10 % of this number. In a recent study **Brass (1985)** has compared the ability of normal platelets and thrombasthenic platelets to exchange Ca++. It was found that thrombasthenic platelets had a much reduced ability to exchange Ca++. Moreover normal platelets exposed to agents that dissociate the IIb/IIIa complex also display a reduced Ca++ exchange. The deficiency in the exchange of Ca++ was predominantly in the Ca++ influx component rather than Ca++ efflux. Hence the glycoprotein complex IIb/IIIa may comprise or be assosiated with the Ca++ channel.

In the present study the maximum elevation in $[Ca^{++}]_i$ was slightly less than 1 μM. Since the principle contribution to changes of $[Ca^{++}]_i$ is a passive influx of $[Ca^{++}]_o$ then opening a Ca++ channel in the plasma membrane, in the presence of an extracellular Ca++ concentration of 1 mM could elevate $[Ca^{++}]_i$ to greater than 1 mM, infact to 10 mM given a membrane potential of - 58 mV.

Therefore there must exist a mechanism which limits the extent of Ca++ influx. In molluscan neurons **Tillotson (1984)** has demonstrated that inactivation of the Ca++ conductance is dependent on the entry of the Ca++ ion itself. Hence the influx of Ca++ may be responsible for closing the gating mechanism.

The platelet clearly posesses efficient Ca++ homeostatic mechanisms. One would expect that were influx of Ca++ to be the principle source of elevated $[Ca^{++}]_i$, then although sequestration of Ca++, by the DTS or by mitochondria, may account for short term Ca++ homeostasis, long term homeostasis would require extrusion of Ca++.

Interestingly in a study designed to determine the subcellular distribution of the platelet Ca++/Mg ATPase the location of this Ca++ pump was predominantly in the vesicle membrane rather than in the plasma membrane. The biochemical events underlying the Ca++ homeostatic mechanisms are unclear. The most potent activator of the Ca++ pump is cAMP however agonists do not apparently elevate the level of this nucleotide.

The role of cyclic GMP, as an activator of the Ca++ pump, has not yet been examined. However cGMP is known to promote the phosphorylation of the 23 Kd protein associated with activation of the Ca++ pump. Agonists have been reported to elevate cGMP though this has not been a universal finding and was not observed in this study. Platelet agonists are known to induce the formation of unsaturated fatty acid peroxides, as a relatively late event following receptor occupancy. It remains a tantilising prospect that such entities are potent activators of guanylate cyclase, and by eliciting the formation of cGMP could play a role in the termination of cellular responsiveness.

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