HUMAN PLATELET ACTIVATION BY ADRENALINE

by

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STATEMENT

Except where acknowledged, the investigations described in this thesis are my own original work.

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To Carl

PREFACE

Adrenaline may facilitate platelet reactions in the normal haemostatic response and, if increased platelet reactivity leads to atherosclerosis, adrenaline could thereby contribute to atherogenesis. Adrenaline levels rise during stress, and in other risk factor conditions for coronary artery desease. The action of adrenaline by itself on platelets can be inhibited by cyclooxygenase inhibitors, but the ability of adrenaline to potentiate the response to other agonists is not completely inhibitable by these drugs. The mechanisms of potentiation of platelet responses by adrenaline is the subject of this thesis.

Chapter 1 emphasises the platelet as a subject of greater interest in the pathogenesis of atherosclerosis and its complications and outlines its structure and functions in relation to this. The known effects of adrenaline on the platelet, together with current understanding of pathways of platelet activation are discussed. Chapter 2 describes the materials and methods employed in the course of the investigations carried out.

Chapters 3-7 describe the experimental work undertaken to investigate some aspects of adrenaline-platelet interaction. The exploration of the effects of multiple agonists, including adrenaline and noradrenaline, is described in Chapter 3. Investigation of the mechanism(s) of potentiation by adrenaline is commenced by the verification in Chapter 4 of previously published evidence

that adrenaline stimulates both a prostaglandin-independent and a prostaglandin-dependent pathway.

Chapter 5 reports the unexpected finding of stimulated thromboxane formation, by adrenaline, in the presence of the phospholipase A_2 inhibitors, BPB and TPCK, suggesting involvement of adrenaline in arachidonic acid release by a pathway not involving phospholipase A_2 enzymes. The hypothesis is put forward that adrenaline in the presence of other agonists stimulates the release of arachidonic acid from diacylglycerol by diacylglycerol lipase.

Chapter 6 examines the characteristics of platelet aggregation, release of nucleotide, thromboxane formation and phospholipid changes due to exogenous phospholipase C. In the presence of BPB, exogenous phospholipase C is apparently more active as indicated by the increased breakdown of PC, but does not result in further PA production, indicating that perhaps diacylglcerol lipase is more active in these circumstances. Thrombin-stimulated endogenous phospholipase C did not, however, behave in this manner in the presence of BPB.

The possibility was considered that adrenaline influences PI turnover and thereby achieves potentiation. Studies of phospholipid metabolism were therefore undertaken. The effects of adrenaline on ADP- and thrombin-induced changes in phospholipids is the subject of Chapter 7. The development of a paper chromatographic technique for the estimation of PI and PA turnover and amounts is reported.

Chapter 8 reviews and discusses the findings of Chapters 3-7 and attempts to establish hypotheses for the mechanism(s) by which adrenaline influences platelets to ensure that they respond more effectively to other agonists. Phospholipid changes due to adrenaline proved difficult to demonstrate. Calcium redistribution was not explored in this thesis and there obviously remains, many aspects of the adrenaline-platelet interaction which need to be investigated.

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ABBREVIATIONS

ACD	acid citrate dextrose
ADP	adenosine diphosphate
ASA	acetylsalicylic acid
ATP	adenosine triphosphate
BHT	butylated hydroxytoluene
BPB	bromophenacyl bromide
Bt_2c'AMP	dibutryl cyclic adenosine monophosphate
betaTG	beta-thromboglobulin
BW755C	3-amino-1-[M-(trifluoromethyl)-phenyl]-2-pyrazoline
Ca^{2+}	calcium ions
c'AMP	cyclic adenosine monophosphate
c'GMP	cyclic guanine monophosphate
DMSO	dimethylsulphoxide
HETE	12-hydroxyeicosatrienoic acid
HPETE	12-hydroperoxyeicosatrienoic acid
5HT	5-hydroxytryptamine (serotonin)
KA	potassium arachidonate
NSB	non-specific binding
PA	phosphatidic acid
PAF	platelet activating factor
PC	phosphatidylcoline
PDGF	platelet-derived growth factor
PE	phosphatidylethanolamine
PF_4	platelet factor 4

PGE_1, D_2	prostaglandins E_1, D_2
PGI_2	prostacyclin
PI	phosphatidylinositol
PPP	platelet-poor plasma
PRP	platelet-rich plasma
PS	phosphatidylserine
RD	reagent diluent
SD	standard deviation
SG	silica gel
SPH	sphingomyelin
TPCK	tosylphenylalaninechloromethylketone
$TXA_2.B_2$	thromboxane A_2, B_2
TMB-8	8-(N,N-diethylamino)-octyl-3,4,5-trimethoxy-benzoate

CHAPTER 1

GENERAL INTRODUCTION

1.1 HISTORICAL BACKGROUND

The study of blood platelets commenced over one hundred years ago. In the early nineteenth century, as microscopes improved, so did the observations of blood components. In 1842, Donné reported smaller 'globules' than the red and white elements, although he erroneously placed their origins in the lymph. At about the same time, two independent English medical practitioners, Gulliver and Addison noted minute spherules in the blood. Both these men also reported the association of these particles with fibrin, and made the first drawings of a platelet-fibrin clot. There was still confusion over the existence of platelets for several years however, with the result that only red and white cells were investigated for involvement in coagulation and inflammation.

With the use of anticoagulants, platelets were more easily observed. Zimmerman (1846) repeated the work of Simon (1842) to establish the presence of colourless bodies, 2-5μ in size but thought they came from the lymphatic system and were the origin of the red cell.

In 1850, Wharton Jones described colourless corpuscles incorporated in a mass which adhered to blood vessel walls and blocked flow of blood in the web of a frog's foot. Through the remainder of the nineteenth century there were several more developments concerning the role of platelets in clot formation. Osler (1874) illustrated single elements in the circulating blood which had the capacity to

aggregate when the blood escaped from the blood vessel. Hayem (1878) added another function for platelets, namely 'regenerating' the blood after haemorrhage. Bizzozero (1882) seems to be the first to have observed circulating platelets in living animals, and demonstrated conclusively that the white portion of thrombi consisted almost exclusively of platelets, then called "petites plaques". These observations were later confirmed and extended by Eberth and Schimmelbusch (1885) and Laker (1889).

In 1906, Wright determined the genetic relationship of the platelet to the megakaryocyte, denying all theories of platelets originating from the white cell or as an early stage of the red cell. There was still doubt about the validity of these early observations, however, until the mid-twentieth century.

Even before the participation of platelets in thrombus or haemostatic plug formation was established, mural thrombi were thought to be involved in the development of atherosclerosis. In 1852, von Rokitansky proposed that atheroma resulted from the degeneration of a deposit of blood products, largely fibrin. Virchow (1856) and later, Hogerstedt and Nemser (1897) proposed that thrombi might contribute to the development of atherosclerosis, a theory revived in 1936 by Clark, Graef and Chasis and by Duguid in 1948. A large part of current work on platelets is directed towards determining the mechanism whereby platelets and thrombi contribute to the inception and progression of atherosclerosis.

1.2 PLATELET STRUCTURE

The platelet is normally discoid, 2-4μ in diameter, and is formed by the fragmentation of megakaryocyte cytoplasm. The circulating platelet count in man is about $2x10^8$/ml with a turnover rate of about $3.5x10^7$/ml/day (Harker and Finch, 1969). Its structure is far from simple.

1.2.1 The Platelet Surface

The platelet surface consists of the plasmatic atmosphere, an ill-defined cloud of plasma proteins, and the plasma membrane proper which resembles that of other cells. This membrane possesses several exceptional features enabling the platelet to carry out its unique functions. The plasmatic atmosphere has not been well characterized but it has been proposed as the cause of the observed differences in behaviour between platelets in plasma and washed platelets in *in vitro* studies(Ardlie and Han,1974).

The glycoproteins of the outermost membrane layer, the glycocalyx, contribute largely to the responsiveness of the platelet. While more than 30 electrophoretically different glycoprotein bands have been identified (Berndt and Phillips, 1981a) only five have become the subject of detailed investigation. The glycoproteins Ib,IIb,III and IV are present in particularly high concentrations.
Several lines of evidence are consistent with glycoprotein Ib/glycocalicin being the thrombin receptor on human platelets (Okumura and Jamieson,1976; Okumura *et al*.,1978; Ganguly, 1977; Ganguly and Gould, 1979). There is

considerable evidence that glycoproteins IIb and III are subunits of a single glycoprotein, and together may participate in the aggregation process (Phillips et al., 1980). No biological function has yet been ascribed to glycoprotein IV. The distinguishing feature of the single chain polypeptide, glycoprotein V is that it is the sole thrombin substrate on the surface of intact, washed human platelets (Berndt and Phillips, 1981a,b). Purified glycoprotein V behaves as a thrombin substrate equally with that on intact platelets (Berndt and Phillips, 1981b,c).

The plasma membrane is similar to other cell plasma membranes in having an apparent trilaminar structure of protein and lipid. It differs from other cells in possessing many invaginations and 'knobs' (Feagler et al., 1974) as seen in freeze-etched fixed platelets. The membrane extends into the open canalicular system or surface connecting system and the inner surface of the membrane contains numerous fibrillar structures.

Compositional analysis of the isolated plasma membrane indicates it is composed of 52% lipid, 36% protein and 7% carbohydrate which can be divided into two lipoglycoprotein subfractions of two different densities (Barber and Jamieson, 1970). The 52% lipid is further divided into 42% phospholipid and 10% cholesterol. The carbohydrate consists of glucose, galactose, mannose, hexosamine, sialic acid and fucose. The sialic acid contributes to the negative charge on the platelet surface (Seaman and Vassar,

1966) and the removal of terminal sialic acid residues from membrane glycoproteins and glycolipids by neuraminidase slightly enhances platelet responses to aggregating agents (Greenberg et al., 1975).

Phospholipids of platelet membranes include: sphingomyelin(SM), phosphatidylcholine(PC), phosphatidylethanolamine(PE), phosphatidylserine(PS) and phosphatidylinositol(PI). Platelet membranes(including outer plasma membrane and membrane from intracellular structures such as dense tubular system) contain, relative to other phospholipids, more PI and PC than does the granule fraction (Marcus et al.,1969). Only minor amounts of PS and PE are present at the exterior of the plasma membrane, but platelet activation may increase accessibility of PE(Schick et al.,1976; Rittenhouse-Simmons et al.,1976). Exposure of phophatidylserine by collagen or thrombin increases the procoagulant activity of the platelet (Bevers et al.,1983) and enhances the conversion prothrombin to thrombin. Perret et al.(1979) published the proportions of the phospholipids in the exterior of the outer membrane to be: PC 23%, PE 11%, PI 10%, PS 4.5% and SM 70%, using enzymes which hydrolyzed phospholipids without causing lysis of the platelets. Phospholipid metabolism and its role in platelet function will be discussed later (1.5.4).

Procoagulant activities of platelets, reside in the membrane. One of these activities has been termed platelet factor 3, and this activity resides in phospholipids(mainly

phosphatidylserine) on the inside leaf of the bilayer membrane(Hemker et al.,1983). Because platelet lipids have this role in blood coagulation, and because phospholipids have been shown to participate in the aggregation and secretion process, platelet lipids are an important subject of research in the platelet field. Platelet lipids are indeed closely linked to platelet reactivity. The role of arachidonic acid metabolism in platelet activation, in particular, will be discussed (1.5.3).

Platelet cholesterol content reflects the initial composition of the megakaryocyte from which platelets are derived, but there can also be some exchange with lipoprotein-cholesterol in the platelet environment (Derksen and Cohen, 1973). Platelets cannot synthesize cholesterol. Carvalho et al. (1973) found that patients with Type II hyperbetalipoproteinemia have enhanced platelet responses to adrenaline, collagen and ADP but the mechanism for this cholesterol influence has not been established. Shattil et al. (1975) found that incubation of platelets with cholesterol-rich liposomes raised the membrane cholesterol level and increased 35-fold the sensitivity of platelets to adrenaline and ADP. Correspondingly, a reduction in membrane cholesterol was associated with an 18-fold reduction in platelet sensitivity to adrenaline.

Of further interest are the findings of Hassall et al.(1983) who established a correlation between low density lipoproteins and total cholesterol concentrations with

sensitivity to aggregation by adrenaline but no other agonists. A possible mechanism for the influence of cholesterol has been suggested by Kramer et al.(1982) who found that elevated platelet cholesterol content affected thrombin-induced changes in platelet phospholipids with an increased metabolism of [^{3}H]arachidonic acid from phosphatidylcholine. It is proposed that cholesterol affects lipid organization and thus affects phospholipase activity for the liberation of arachidonic acid.

The role of platelet membrane glycolipids in platelet function is unknown. Gangliosides and ceramides have only been the subject of limited experimentation to date.

1.2.2. Microfilaments and Microtubules

The fibre system of the platelets in the cytoplasm supports the discoid shape of unstimulated platelets. Electron micrographs show the circumferential band of microtubules as marginal bundles with long straight sections, possibly under tension (Behnke, 1965; Haydon and Taylor, 1965). The bundle of microtubules never directly contacts the cell wall of the platelet but forces necessary to maintain the discoid shape can be transmitted through submembrane filaments (White, 1972a). The discoid shape is also regulated by intrinsic properties of the surface membrane, by cyclic nucleotides, and by the maintenance of high levels of ATP as will be discussed later (1.5.1).

Platelet stimulation inducing a shape change, is accompanied by a contraction of the ring of microtubules and microfilaments around tightly packed central organelles

(White, 1968). In addition, the dense tubular system is a calcium sequestering site in platelets, resembling the sarcotubules of muscle cells. The channels of the open canalicular system, with their specialized microfilaments, are similar to the transverse tubules of muscle (White, 1972b). The platelet therefore has all the contractile elements necessary to undergo shape change when required. The microtubules and microfilaments are also involved in the release reaction of platelets (MacIntyre, 1976).

1.2.3 Platelet Organelles

The cytoplasm of platelets also contains mitochondria, α-granules, dense bodies, lysosomes, occasional Golgi apparatus and glycogen granules (White and Gerrard, 1978). The number of mitochondria in platelets, however, are comparatively low.

Dense bodies contain serotonin, ADP, calcium and phosphorus; the last two constituents contribute to the electron dense properties of dense bodies (Skaer, 1975). The phosphorus is only partially accounted for by ATP and ADP. Dense bodies also contain large amounts of phospholipid, probably rich in arachidonic acid (Skaer *et al.*, 1976). This was suggested by the finding of arachidonic acid by autoradiography in a structure regarded as a stage in the discharge of dense bodies (Skaer and Skaer, 1979). Some of the dense body contents promote growth of platelet aggregates, and are released from the dense bodies into the platelet environment. Skaer (1981) proposed a mechanism involving movement of dense bodies to

the surface of the platelet where they distort the membrane, fusing with it and then enlarging. The resultant spheroid then releases its contents, leaving behind an empty shell.

The α-granules also participate in the release reaction. Holme et al. (1973) observed the presence of fibrin, and White (1972b) observed nucleoids which originated from the α-granules, in the canalicular system and concluded that fusion of the α-granule membranes with the inner surface of the open canalicular system occurs, allowing the contents to diffuse out. The contents include fibrinogen, platelet factor 4 (anti-heparin factor), beta-thromboglobulin and platelet derived growth factor(PDGF).

Lysosomes contain enzymes including cathepsins, glycosidases, a chemotactic factor for leucocytes and a cationic protein which increases vascular permeability and induces histamine release from mast cells. Lysosomal degranulation is a fairly slow process(Holmsen et al.,1969) and the mechanism is unclear. These diverse inclusions of the platelet and its complex structure enable this cell to have many functions.

1.3 PLATELET FUNCTION

The smallness of the platelet with respect to the other blood cells should not lead to an underestimation of its importance.

1.3.1 Maintenance of Integrity of the Vasculature

Gimbrone et al. (1969) found that capillary integrity, in isolated perfused organs, was better preserved when the perfusate contained intact viable platelets in addition to plasma proteins and the necessary ions. Thrombocytopenia is associated with an increase in capillary fragility. Evidence for the close association of platelets with endothelial cells lining capillaries was obtained by Johnson (1971) in transfusion studies in thrombocytopenic animals. She found that the transfusion of radioactively labelled platelets labelled the capillaries of many organs whereas labelled platelet-poor plasma did not. Ultrastructural studies confirmed the association but could not explain the interaction of platelets with endothelial cells.

The endothelial lining depends on circulating platelets for sustained normal functioning, while the endothelium protects platelets from activation and subsequent aggregation. Tissue culture studies of endothelial cells indicate that a platelet factor promotes replacement of a partial removal of endothelial cells from monolayers. Migration of endothelial cells into the damaged area is enhanced by platelet-rich serum (Wall et al., 1978) though smooth muscle cell migration is more enhanced by platelet factors (Thorgeirsson et al., 1979). Mitogenic responses, as determined by tritiated thymidine incorporation were increased by platelet-rich serum added to pre-confluent monolayers of endothelial cells (Fratkin

et al., 1980). On the other hand, confluent monolayers did not respond in agreement with the work of Thorgiersson and Robertson (1978). Smooth muscle cells again differed from endothelial cells in that they exhibited a mitogenic response whilst confluent. The mitogenic factor found in serum is the platelet derived growth factor from the α-granules (Antoniades et al., 1975; Busch et al., 1976; Antoniades and Scher, 1977). Platelets probably assist in the repair of injured blood vessels by regulating endothelial cell and smooth muscle cell growth. This facility forms the basis of the response to injury hypothesis for atherogenesis(see Section 1.3.4).

1.3.2 Inflammation

Platelets accumulate at sites of tissue damage and aid in the inflammatory response by increasing vascular permeability and by being chemotactic for leucocytes. Nachman et al. (1972) found a permeability factor in the lysosomes associated with histamine release. Chemotaxis of leukocytes could be brought about by several possible candidates. Weksler and Coupal (1973) found that a cationic protein from the lysosomes liberated a chemotactic factor from the fifth component of complement. The lipoxygenase product, 12L-hydroxy-5,8,10,14-eicosatetraenoic acid (HETE) was shown by Turner et al. (1975) to induce leukocyte chemotaxis and Kitchen et al. (1978) showed some chemotactic activity of thromboxane B_2 (TXB_2).

Through the action of proteolytic enzymes, platelets also have the potential to participate in the destruction of connective tissue in the chronic inflammatory disease state (Zucker, 1974). Thrombin releases a considerable proportion of platelet β-gluceronidase, β-N-acetylglucosaminidase and cathepsin, hydrolytic enzymes of lysosomes. Further, antigen-antibody complexes interact with the platelet Fc-receptor and thereby induce the release reaction (Humphrey and Jacques, 1955).

1.3.3 Haemostasis

The most important role of platelets is in the haemostatic process. Platelets initiate the formation of a haemostatic plug by accumulating at the site of vascular injury. These adherent and stimulated platelets then release factors (eg. ADP, TXA_2) promoting aggregation and coagulation. The membrane of the platelet also acts as a cofactor in the clotting cascade.

In order to plug a wound in a blood vessel wall, the platelets have to adhere to the tissue of the wound. Artificial surfaces, (Friedman et al., 1970) and natural vascular surfaces (Baumgartner, 1973) both have the capacity to cause adherence of platelets. Removal of the endothelium of the blood vessel wall exposes the subendothelium collagenous layer. In animal studies, where the endothelial layer is removed artificially, platelets adhere within minutes (Baumgartner and Spaet, 1970; Stemerman and Ross, 1972; Nam et al., 1973). Electron micrographs (Baumgartner et al., 1967; Baumgartner, 1977)

illustrate the adhesion of platelets to the subendothelium.

Degranulation of adherent platelets brings about the formation of a platelet plug by the process of platelet aggregation. An electron micrograph taken 10 minutes after removal of the rabbit endothelium shows platelets which have undergone a shape change. Pseudopodia interlink platelets and those platelets closest to the subendothelial surface show a loss of alpha granules and serotonin storage granules. After forty minutes, all that remains is a thin covering of flattened, degranulated platelets.

Exposed subendothelium also activates coagulation factor XII. The intrinsic pathway of coagulation is further promoted by platelets at several steps when they accumulate at sites of vascular injury (Walsh, 1974). The ADP-stimulated platelet membrane surface provides a suitable surface for the activation of factor XII. The collagen-stimulated platelet membrane activates factor XI. Phospholipids normally found on the interior of the platelet membrane may be exposed by disruption of the platelet or by a specific mechanism called flip-flop where different phospholipids are swapped from inside to outside and vice versa. This mechanism is stimulated by the simultaneous presence of small amounts of collagen and thrombin (Hemker et al., 1983). Exposed platelet membrane procoagulant phospholipids bind factors IXa and VIIIa to form the factor X converting enzyme. They also provide for the interaction of factor Xa and factor Va to form

prothrombinase which forms thrombin. Fibrin formation is thus promoted and the haemostatic plug is consolidated.

White cell accumulation occurs secondarily to platelet adhesion, probably as a result of release of chemotactic substances as mentioned before. These cells could serve to phagocytize cellular debris.

When a blood vessel is intact, the endothelium may counteract platelet activity in several ways. Moncada et al.(1977) found that prostacyclin(PGI_2) production by the arterial wall was highest in the intimal region. Martin et al. (1980) found that PGI_2 prevents platelet aggregation in vivo and possibly promotes disaggregation. PGI_2 has been shown to increase c'AMP levels in platelets which inhibits aggregation (Gorman et al., 1977a). Use of PGI_2-neutralizing antibodies by Steer et al. (1980) indicated that circulating levels of the hormone are insufficient to prevent in vitro aggregation. This may mean that PGI_2 acts in cooperation with other anti-aggregatory factors in vivo.

Other means by which the endothelium may be anti-aggregatory are the production of plasminogen activator activity, heparin-like activity (heparan sulphate), ADP-ase activity and thrombin-binding activity. These factors in addition to the endothelium being a physical barrier between the blood and the underlying connective tissue and smooth muscle cells, make the endothelial lining an important protective organ (Nalbandian et al., 1979).

1.3.4 Aberrant Haemostasis: Thrombosis and Atherosclerosis.

Haemostasis, as summarized above, involves the interaction of platelets, the vessel wall and plasma coagulation factors. Thrombosis may be considered as an aberrant form of the haemostatic process. Atherosclerosis can be considered also as an undesirable reactive response of the blood vessel wall to injury, although there is still controversy over the mechanism of atherogenesis.

The characteristics of atherosclerosis are: localized lesions of the arterial intima which contain smooth muscle cells, fibrous tissue and acculumated lipid, associated with a narrowing of the arterial lumen (Wissler,1976). The lesion commences in the intima mainly around vessel orifices and branches. The intimal thickenings are rich in smooth muscle cells, connective tissue and proteoglycans (Geer and Haust, 1972; Strong and McGill, 1962). Within, and between cells, lipids collect, particularly low density lipoproteins (LDL) and cholesterol crystals. A fibrous cap overlies the resultant plaque.

No single theory of atherogenesis has been proven. It is, however, undisputed that advanced atherosclerotic lesions often have thrombi associated with their surfaces. Histological and morphological evidence indicates that intimal thickenings have incorporated thrombus material (Mustard and Packham, 1975). The platelet-endothelial interaction is therefore of considerable importance in the development of atherosclerosis, be it biochemical or biophysical.

The response to injury hypothesis of Ross and Glomset (1976) postulates that injury to the endothelium gives rise to events which, if they occur repeatedly, cause an atherosclerotic lesion. The injury to the endothelium may take the form of desquamation, lysis or changes in the permeability characteristics of the cells. There could then be a focal adherence of platelets at the injured site, followed by release of platelet granule contents including PDGF which enhances proliferation of arterial smooth muscle cells(Section 1.3.1). If there is also platelet aggregation, there would be even further release. This hypothesis is not entirely new, but rather a modification of that documented by Duguid (1949) who followed the line of thinking from several workers as previously mentioned (Section 1.1).

According to the response to injury hypothesis, PDGF provides a stimulus for focal migration and proliferation of smooth muscle cells. These cells may be derived either from the media of the artery wall or from pre-existing cells within the intima. These proliferative cells are stimulated to also synthesize connective tissue, including collagen, elastic fibre proteins and glycosaminoglycans. Lipids accumulate both inside and outside cells, particularly if plasma lipoprotein levels are raised, or the endothelium is damaged.

An important feature of the response to injury hypothesis is that the 'injury' must occur on a chronic or recurrent basis. The response to single injury is a normal

haemostatic response, such lesions regressing and disappearing once repair is complete (Ross and Harker, 1976). Repetition of such injuries could conceivably occur in the presence of several known risk factors for coronary heart disease. Major risk factors include smoking, hypertension and hypercholesterolaemia.

Hypertension could lead to chronic damage of endothelial cells at bifurcations of arteries where also, platelet reactivity may be increased. Lipoproteins have been shown to have some ability to stimulate proliferation of smooth muscle cells (Fisher-Dzoga et al., 1974) and to inhibit PGI_2 production (Nordoy et al.,1978). Lipoproteins are also incorporated into the atherosclerotic plaque, the consequences of which are uncertain. Hypercholesterolaemia was shown by Ross and Harker(1976) to decrease platelet survival, probably by loss of endothelium sustained by chronic hypercholesterolaemia. Also, platelet reactivity is increased by increased cholesterol incorporation (Shattil et al., 1975) and by increased saturated fats in the diet (Jakubowski and Ardlie, 1978). Smoking, which is another risk factor, has been found to affect the haemostatic system in several and complex ways. For example, it increases the sensitivity of platelets to ADP (Hawkins, 1972). Aryl hydrocarbons (premutagens) from cigarettes are taken up and carried by the same fractions in the plasma as cholesterol, namely the lipoproteins (Benditt, 1976). This would provide a mechanism for introduction to the vessel wall of a mutagen responsible

for induction of proliferation. Also, Mustard and Murphy (1963) proposed that cigarette smoke damages the endothelium.

Another hypothesis of major interest in atherogenesis is the monoclonal hypothesis. Benditt and Benditt (1973) discovered that the cellular composition of atherosclerotic plaques is monoclonal, with only a small percentage polyclonal, as characterized by enzyme analysis. Similar findings have been described by Pearson et al. (1977). According to this hypothesis atherosclerosis is initiated by a mutation caused by chemicals, possibly cigarette smoke, radiation or viruses. The cells with a selective advantage then proliferate. Further development of the disease can occur when platelets then form thrombi on these thickened regions. This hypothesis also proposes 'injury' as an initiator and includes platelet involvement in its development.

The earlier theories concentrated on the lipid nature of the lesions, rather than their cellular nature. In most of the current theories, lipid accumulation is regarded more as a secondary event. More emphasis is placed on the origin of cellular proliferative stimuli. Research is concentrated on the possible biochemical alterations in the endothelium and the stimuli responsible for modification of the smooth muscle cells. The importance of platelets in atherogenesis lies in their relationship to endothelial and smooth muscle cell behaviour.

The work reported in this thesis concentrates on the mechanisms of platelet activation keeping in mind the involvement of platelets in thrombosis and atherosclerosis.

1.4 PLATELET INTERACTIONS

The studies of platelet interactions with various blood and vessel wall components, have been mostly carried out, for convenience, *in vitro*. Biogenic amines, thrombin, connective tissue and ADP are *in vivo* compounds which are known to interact with platelets.

1.4.1 Catecholamines

Baseline plasma levels of catecholamines have been estimated at 3.4nM for noradrenaline and 0.64nM for adrenaline (Dimsdale and Moss, 1980). During public speaking, adrenaline levels increase twofold, and during moderate physical excercise, noradrenaline levels increase threefold (Dimsdale and Moss, 1980). Roizen *et al*. (1975) found catecholamine levels to change dramatically with changes in body temperature in rats. Catecholamine levels are capable of changing rapidly to meet acute body reactions to environmental and activity changes.

Catecholamines accumulate in pig platelets (Born *et al*., 1958) against a concentration gradient, but the affinity of this transport system is unknown. Interpretation of data on the subject is difficult. Experiments performed on plasma without pH control may have artificially affected uptake results. Similarly, data on

washed platelets for accumulation of catecholamines may be artifactual.

The stimulation of platelets by catecholamines produces a series of reactions. It is difficult to separate pure catecholamine responses from those which may be induced via secondary stimulation by, for example, ADP. Adrenaline has been shown, however, to interact with its own specific cellular receptor (Scrutton and Wallis, 1981), whereas released ADP may mediate secondary aggregation. Studies using selective agonists and antagonists have demonstrated that stimulation of an α-adrenoceptor is responsible for initiation of aggregation and secretory responses of human platelets to adrenaline. Radioligand and physiological response studies suggest that both the aggregatory and pro-aggregatory responses of human platelets to adrenaline are mediated by α_2-adrenoceptors (Grant and Scrutton, 1980b; Clare et al., 1984). More recent studies, however, suggest that the present understanding of the relationship between receptor occupation and functional responses of platelets may be incorrect (Swart et al.,1984). Although inhibition of adenylate cyclase is widely accepted as the typical stimulus-response coupling mechanism for α_2-adrenoceptors, this cannot explain induction of platelet aggregation by adrenaline and therefore unique α_2-adrenoceptors may be involved in the aggregatory response to adrenaline (Clare et al.,1984).

There has been some disagreement as to whether adrenaline causes platelets to change shape. Mills (1973)

based on observations on ATP consumption in combination with aggregation tracings, found no shape change-associated decrease in the platelet adenylate energy charge and concluded that adrenaline does not induce a shape change in human platelets. Patscheke (1980) observed an adrenaline shape change in recordings for washed platelets suspended in a medium containing 1mM calcium. The so-called shape change due to adrenaline certainly differs from that caused by ADP.

Adrenaline, when capable of causing aggregation in vitro, induces two waves of aggregation in human platelets: a primary and a secondary response. The secondary response is associated with the release reaction and a minimal amount of thromboxane production. Arachidonic acid, released from phospholipids, is metabolized via the endoperoxides to thromboxanes and several other products. Thromboxane A_2 is a potent aggregating compound. Arachidonic acid is also metabolized by the enzyme lipoxygenase but the roles for the products of this pathway are uncertain. There are several questions so far unanswered in regard to the mechanism of induction of these responses to adrenaline, and the connections between the two phases.

One point of confusion arises from the choice of anticoagulant for blood collection for the preparation of the platelets. Primary aggregation occurs in both citrated and heparinized plasma, while it is increased in the latter (O'Brien et al., 1969). Secondary aggregation is the

predominant response to adrenaline in citrated plasma, and is dependent on arachidonic acid metabolism by cyclo-oxygenase to form thromboxane A_2 (Best *et al.*, 1980). Both secretion of serotonin (5-hydroxytryptamine) and thromboxane B_2(TXB_2), the stable end-product of TXA_2, are inhibited by 100μM acetylsalicylic acid (aspirin) which irreversibly acetylates cyclooxygenase. The second phase of the adrenaline response is therefore totally abolished by aspirin while the small primary response remains unaffected.

It is not known whether this secondary aggregation is important *in vivo* as it could be purely an artifact of citrated plasma (see 2.1.1.2.). (See Chapter 3 for a comparison of platelet reactivity in heparin vs. citrate.) The involvement of adrenaline and noradrenaline in the reactivity of platelets and thus thrombosis and atherosclerosis is an important area of investigation. It is possible that the major importance of adrenaline lies in its preparation of platelets to respond to other agonists. Adrenaline and noradrenaline are known to potentiate the aggregation response to other agonists *in vitro*. Ardlie *et al*. (1966) first demonstrated this and it was studied in detail by Mills and Roberts (1967). In particular they studied adrenaline potentiation of the response to nucleotides. Since then, several authors have observed adrenaline potentiation of the response to thrombin, collagen, serotonin and vasopressin (Baumgartner and Born, 1968; Thomas, 1967; Grant and Scrutton, 1980a).

Of further interest is the inability of aspirin to prevent adrenaline potentiaton of ADP-induced aggregation. (Rao et al., 1980a). The alteration of the platelet involved in potentiation is at the receptor-membrane level (Rao et al., 1980b; Rao et al., 1981a). It was thought that the effects may be due to changes in calcium mobilization because adrenaline potentiation was inhibited by verapamil,a calcium 'antagonist' (Owen and Le Breton, 1980). It has subsequently been shown, however, that verapamil is an α-adrenergic antagonist(Barnathan et al., 1982) with no receptor sub-type specificity as studied in other tissues(Motulsky et al., 1983). Adrenaline and noradrenaline depress the basal activity of adenylate cyclase in human platelet lysates (Moskowitz et al., 1971; Salzman and Levine, 1971) and lower prostaglandin E_1-induced increases in cyclic AMP in intact platelets (Harwood et al., 1972; Haslam and Taylor, 1971; Robinson et al., 1969) but adrenaline does not depress the level of cyclic AMP in resting platelets nor can the adrenaline response be enhanced by addition of an inhibitor of adenylate cyclase (Haslam et al., 1978a,b). An alternative mechanism of adrenaline potentiation in vitro must be sought.

Adrenaline is also of interest because platelets from human donors exhibit wide variability in their response to this agonist. Sensitivity to adrenaline could conceivably be related to thrombotic tendencies, but such a correlation needs to be established. There is also a need to establish

the physiological role of adrenaline in platelet reactivity. Detailed reviews of recent work on the mechanisms of adrenaline action will be given in later chapters, appropriate to the particular study undertaken. See also Section 1.5 for discussion on calcium,c'AMP and phospholipids in relation to mechanisms of platelet responses.

1.4.2. Serotonin (5-hydroxytryptamine, 5HT)

Serotonin is actively and passively taken up by platelets and stored in the dense granules, where it is inaccessible to the destructive enzyme monoamine oxidase, in preparation for release (Paasonen, 1965). These granules also contain ADP, ATP, magnesium, calcium and other biogenic amines. The interaction of platelets *in vitro* with serotonin commences with a shape change, first observed by O'Brien and Heywood (1966) although aggregation is not necessarily a consequence of this event.

Human platelets, prepared in either citrate or heparin, can be aggregated by micromolar concentrations of serotonin (O'Brien *et al.*, 1969). This aggregation is generally small and reversible, and high concentrations are self-inhibitory (Baumgartner and Born, 1968). Variations in ionized calcium levels can, under certain conditions have dramatic effects on the platelet responsiveness to serotonin. Addition of calcium to citrated human platelets increases the initial velocity of serotonin-induced aggregation but does not affect the maximal response

(O'Brien et al., 1969). Aggregation to serotonin is calcium dependent to this extent.

A small percentage of human donors is found to exhibit a secondary response to serotonin with an associated release of adenine nucleotides. Some donors have serotonin -insensitive platelets.

Serotonin, like adrenaline, can potentiate the platelet response to other agonists (Baumgartner and Born, 1968). There are serotonin receptors on the platelet of heterogeneous types, either related to shape change or serotonin uptake (Drummond, 1976).

1.4.3. Thrombin

Since only a few platelets would be in contact with collagen at a wound surface, other agents are expected to be involved in consolidating a platelet mass in the formation of a haemostatic plug. Thrombin is formed at the sites of injury in blood vessels. Thrombin is therefore of interest in in vitro studies.

The effects of thrombin on platelets include an increase in phosphatidylinositol (PI) turnover and an increase in protein phosphorylation (Lyons et al., 1975). Diacylglycerol production from PI hydrolysis has been reported to be coupled to a protein kinase thought to participate in the release reaction through phosphorylation of a 40,000dalton M.W. polypeptide (Kawahara et al., 1980). Breakdown of phosphatidylinositol by phospholipase C due to thrombin stimulation, is associated with arachidonate release (Rittenhouse-Simmons, 1979; Bell et al., 1979). If

the metabolism of this arachidonic acid is blocked by indomethacin, aggregation induced by thrombin still occurs but with an increased production of phosphatidic acid, an intermediary in the phosphatidylinositol cycle (Lapetina et al., 1978). Indomethacin also inhibits diglyceride lipase responsible for arachidonic acid release (Rittenhouse-Simmons, 1980). Thrombin also stimulates arachidonic acid release from phosphatidylcholine via the activity of phospholipase A_2 (Bills et al., 1977) so that there are two potential sources of arachidonic acid release due to thrombin. Prostaglandin synthesis occurs subsequent to thrombin activation of arachidonate release (Hamberg et al., 1974).

Thrombin inhibits adenylate cyclase by three concurrent mechanisms: by interaction with its receptor, by mediation via thromboxanes or endoperoxides and by an increase in the level of free calcium in the cytosol(Feinstein et al., 1981). Thrombin binding to its receptor behaves more like a hormone than an enzyme, expressing negative cooperativity for further binding (Majerus et al., 1976). These highly specific receptors bind thrombin with high affinity, ensuring platelet involvement in clot formation. In addition, some thrombin-induced responses, aggregation and dense granule secretion do not require the continuing presence of thrombin on its receptor (Holmsen et al., 1981).

1.4.4. Connective Tissue

A wound in a blood vessel exposes tissues not normally in contact with the blood. Platelets have been found to adhere to connective tissue underlying the endothelium, the first step in haemostatic plug formation. The major components of the connective tissue are collagen, elastin, glycoprotein and proteoglycan. Collagen achieves aggregation of platelets *in vitro* while elastin has been found not to induce aggregation (Hoffman, 1975). Glycosaminoglycans may enhance aggregation (Muir and Mustard, 1968). The enormous amount of work on platelet-collagen adhesion and aggregation will not be included in this introduction.

1.4.5. Adenine Nucleotides

Adenosine diphosphate (ADP) and adenosine triphosphate (ATP) are stored within the platelet in dense granules for release during the release reaction. ADP and ATP located elsewhere in mitochondria, the cytosol and membranes are available for metabolic processes. Platelets challenged with radioactive adenosine, exhibit the label in this metabolic pool, while no label is found in the releasable nucleotides (Holmsen *et al.*, 1969).

There is a critical level of metabolic ATP required for the platelet shape change, aggregation and the release reaction (Holmsen *et al.*, 1974; Holmsen, 1975). Stimulated platelets have an increase in the activity of key enzymes of glycolysis and oxidative phosphorylation as energy is consumed.

The release of nucleotides serves to consolidate a thrombus, as new platelets are challenged and activated by ADP. Studies using ADP *in vitro* show that platelets undergo a shape change almost immediately in response to ADP (Born, 1970) and in the absence of external calcium. The platelets are then able to aggregate, with the release reaction occuring secondarily.

Little is known of the mechanism of induction of shape change, aggregation and release due to ADP. *In vitro* studies illustrate that the shape change is immediate and rapid, with a maximum change occurring in less than thirty seconds. The contractile elements of the platelet immediately beneath the plasma membrane can be rapidly activated following calcium loss from the dense tubular system or other membranes (Behnke, 1970). The work of Lloyd *et al*. (1973a,b) indicates that ADP-induced shape change is associated with phosphatidic acid production.

Calcium is mobilized during shape change, a process which includes psuedopod formation and granule centralization (Le Breton *et al.*, 1976). The calcium may control contraction in platelets through calcium-dependent myosin phosphorylation reactions (Hathaway and Adelstein, 1979) involving ATP bound to actin (Crawford, 1976). The concentric ring of microtubules becomes disorganized, but can become prominent again, extending into the pseudopodia which form during activation. The platelet is then ready for platelet-platelet adhesion.

ADP can induce the release reaction and prostaglandin production but it is not clear whether these events occur in parallel with secondary aggregation, or are the cause of secondary aggregation. Experiments with platelets from patients with storage pool deficiency, deficient in dense granule ADP, show that secondary aggregation is abolished in the absence of endogenous ADP (Weiss, 1975). Lages and Weiss (1980) reported two patients with storage pool deficiency who exhibited biphasic aggregation responses to ADP and adrenaline but these patients could also secrete ADP, despite the deficiency. These authors suggested that secondary aggregation responses may be mediated by both thromboxanes and ADP secreted from platelets.

1.5 MECHANISMS OF PLATELET RESPONSES

1.5.1. The Necessity for Calcium

Platelet activation involves a shape change, the release reaction, prostaglandin metabolism and aggregation. These steps require energy, membrane changes and contractile function, all of which have been thought to be calcium-dependent processes. The manner in which calcium is utilized is less certain.

The platelet shape change induced by ADP or the ionophore A23187 (an agent known to facilitate divalent cation transport across membranes) is associated with a redistribution of intraplatelet divalent cations, as shown by the use of a fluorescent-chlortetracycline probe (Le

Breton et al., 1976). The contractile mechanism involved in constricting the band of microfilaments during the shape change is activated by calcium (Hanson et al., 1973). Calcium is thought to participate in the disappearance of the equatorial bundle of microtubules. Calcium controls the platelet contractile protein, thrombasthenin, through its effect on ATPase activity in a similar way to muscle proteins.

Calcium ionophores also stimulate aggregation and release (White et al., 1974). The regulation of cytoplasmic calcium is thought to control release of arachidonic acid from phospholipid substrates by phospholipase A_2 and thus calcium governs the generation of endoperoxides and thromboxanes from these sources (Rittenhouse-Simmons and Deykin, 1978; Gerrard et al., 1978a). On the other hand, phospholipase C does not have the same requirement for calcium (Billah et al., 1980). The phosphatidate specific phospholipase A_2 described by Billah et al. (1981) requires calcium for the release of arachidonic acid but not to the same extent as the phospholipase A_2 which degrades phosphatidylethanolamine and phosphatidylcholine.

Rittenhouse (1984) concluded that phospholipase C is not activated by calcium and suggested that stimulation is totally dependent upon a receptor-coupled event, possibly via cyclooxygenase products and ADP. The subsequent events of this calcium-independent pathway of platelet activation involves protein kinase C (Simon et al.,1984). Calcium may

influence the phosphatidylinositol cycle initiated by phospholipase C by inhibiting the resynthesis of phosphatidylinositol, causing the accumulation of phosphatidic acid (Lapetina *et al.*, 1981b). Cyclic AMP directly opposes this action.

Dibutyryl cyclic-AMP($Bt_2c'AMP$) and 8-(N,N-diethylaminooctyl-3,4,5-trimethoxybenzoate (TMB-8) oppose arachidonate release. $Bt_2c'AMP$ is thought to compartmentalize calcium and TMB-8 to immobilize calcium, both restricting the availability of calcium for enzyme activation, the latter's specificity albeit doubtful (Simpson *et al.*, 1984). These agents also cause inhibition of thrombin-induced release of serotonin, which can be overcome by the addition of calcium. Perhaps the enzymes of arachidonic acid conversion to thromboxanes are also calcium-dependent since they are inhibited by TMB-8 (Shaw,1981).

The work of White *et al.*(1981) demonstrated a calcium-dependent calcium-binding regulator protein, calmodulin, in platelets. Localization using immunofluorescence was inconclusive, giving general staining. It has been suggested that calmodulin controls phospholipase A_2 activity, and thus arachidonate release (Rao *et al.*, 1980b; Vanderhoek and Feinstein, 1979) but the phenothiazines used as a basis for these studies inhibit other enzymes (Schatzman *et al.*, 1981) and may be α-receptor blocking agents (Cocks *et al.*, 1981). More recent evidence indicates that calcium regulation of phospholipase A_2 is independent of calmodulin (Withnall *et al.*, 1984).

The development of the fluorescent intracellular calcium indicator, quin 2, has provided an important tool to examine in detail the role of calcium in platelet responses to stimuli (Tsien et al.,1982). Quin 2 is added as an ester that readily penetrates platelets, is hydrolized intracellularly and binds calcium. The fluorescence of quin 2 increases when it is complexed with calcium. Studies with quin 2 show that several natural agonists, in the presence of 1mM external calcium, cause cytoplasmic free calcium to increase by both stimulated calcium entry across the plasma membrane and discharge of calcium from intracellular stores (Rink et al., 1982; Hallam et al., 1983a; Feinstein et al., 1983; Kawahara et al., 1983a; Hallam et al., 1984a; Hallam et al., 1984b). When calcium was omitted from the suspending medium, thrombin and other agonists evoke responses when the cytoplasmic free calcium remains near basal levels (Rink et al., 1982; Hallam et al., 1984a; Hallam et al., 1984b). The occurrence of shape change and secretion at basal levels of calcium has been attributed to the ability of diacylglycerol, a product of phosphoinositide hydrolysis, to initiate responses in platelets (Rink et al., 1983) (see 1.5.4). More recently, the photoprotein, aequorin, has been utilized to measure ionized calcium in platelets (Johnson et al., 1985) and this has drawn attention to some potential problems with quin 2 which nonetheless remains a useful indicator of calcium elevation in platelets.

Calcium modulation at the membrane level has been suggested as the means by which adrenaline acts as a potentiator of platelet reactions (Rao et al., 1981a). It has been proposed that calcium participates in the initiating events of adrenaline stimulation of platelets (Owen et al., 1980). With the ^{45}Ca-assay technique, Owen and LeBreton (1980) found that low concentrations of ADP promote the ability of adrenaline to stimulate an increase in membrane permeability to calcium. If, on the other hand, adrenaline is added to the platelets first, no increased calcium uptake is measured. These workers then proposed that some kind of calcium involvement in the potentiated ADP-response on the basis of inhibition of the response by verapamil. As has been mentioned, however, verapamil also acts as a α_2-adrenoceptor inhibitor (Barnathan et al.,1982). (See Section 1.4.1)

Clare and Scrutton(1984) concluded that calcium uptake did occur but as a consequence of secretion due to adrenaline and other agonists. They found that blockade of secretion by aspirin prevented radioactively labelled calcium uptake. Hallam et al. (1983b) also found that adrenaline induces no increase in cytosolic calcium concentration in platelets treated with aspirin. On the other hand, Brass and Shattil(1982) did find that both ADP and adrenaline increased the amount of calcium bound to the platelet surface membrane, despite no net influx of calcium, and without the occurrence of secretion. Hence, there is conflicting evidence as to whether adrenaline induces an

aggregatory response by enhancing calcium influx into human platelets. The involvement of calcium in the pro-aggregatory response to adrenaline has not been investigated in any detail.

1.5.2. Control by c'AMP(cyclic adenosine 3',5'-monophosphate)

The actions of several inhibitors of platelet aggregation and release are mediated by c'AMP (Haslam et al., 1978a). c'AMP is thought to decrease the concentration of calcium ions in the platelet cytosol, probably by activation of a calcium pump. Käser-Glanzman et al.(1977) reported that under certain conditions, addition of c'AMP with platelet c'AMP-dependent protein kinase, can increase the initial uptake of calcium by a platelet membrane fraction containing both plasma and intracellular membranes. c'AMP promotes movement of calcium ions into the dense tubular system or across the plasma membrane or both, and this can be mimicked by dibutyryl cyclic AMP(Bt_2 c'AMP).

Both PGE_1 and PGD_2 were used in early studies to increase adenylate cyclase activity, but PGI_2 has subsequently been found to be far more potent, inhibiting platelet aggregation at nanomolar concentrations (Moncada et al., 1976). As PGI_2 is produced by the endothelial lining of blood vessels, it is thought to be a major regulatory component of the haemostatic process.

Isoproterenol causes increased adenylate cyclase activity by a weak β-adrenergic effect. Platelet

aggregation is further inhibited by c'AMP phosphodiesterase inhibitors e.g. papaverine, which prevent breakdown of c'AMP (Mills et al., 1970).

Adenylate cyclase activity is itself modulated by released calcium (Kume and Kariya, 1980). Thus, the attenuation by ADP and adrenaline of increased c'AMP by PGE_1, is likely to be due, not only to receptor-mediated inhibition, but also to calcium-mediated inhibition of adenylate cyclase.

Cyclic guanosine 3',5'-monophosphate (c'GMP) has been found to increase during platelet activation by adrenaline (Jakobs et al.,1974) and by other agonists(Haslam et al., 1978b). Kume and Kariya (1980) found c'GMP had an inhibitory effect on c'AMP-dependent phosphorylation of platelet proteins which could regulate platelet function. Takai et al.(1981) suggested that increased c'GMP levels, caused by thrombin stimulation, lead to feedback inhibition of phosphatidylinositol hydrolysis, presumably through protein phosphorylation.

The interrelationships of the cyclic nucleotides are obviously complex. For example, Steer and Wood (1979) suggested from their observations, that guanine nucleotides mediate either stimulation or inhibition of adenylate cyclase, depending on which binding site is involved. One of these guanine nucleotide sites is thought to interact with the α-receptor to result in enzyme inhibition, while the other interacts with the PGE_1-receptor to cause stimulation. Steer and Wood (1979) also found while adrenaline and GTP individually inhibited

adenylate cyclase slightly in isolated membranes, adrenaline and GTP together act synergistically to cause 50% inhibition of cyclase activity.

Membrane phospholipids may also be involved. In hamster adipocytes, phosphatidic acid, a product of phosphatidylinositol turnover, inhibited methyl xanthine-stimulated c'AMP formation (Schimmel et al., 1980). Phosphatidic acid is formed in platelet membranes upon stimulation by thrombin (Rittenhouse-Simmons, 1979) and is perhaps a means by which thrombin inhibits adenylate cyclase. It is in this area of assigning roles to phospholipids in platelets that attention is currently focussed (See 1.5.4).

1.5.3. Prostaglandins (PG) and the Release Reaction

Secondary aggregation is associated with both the secretion of dense and α-granule constituents, including the aggregating agents 5HT and ADP, and the activation of a pathway for conversion of arachidonic acid to prostaglandins (Smith et al., 1973). Cyclooxygenase converts arachidonic acid to endoperoxides which form thromboxanes (Fig 1.1). The precise role of endoperoxides and thromboxanes so formed is still a matter of controversy. Thrombin and collagen may cause secretion in the absence of thromboxane synthresis whereas ADP induced secretion is dependent on thromboxane synthesis.

Best et al. (1980) concluded that the aggregation process can occur independently of secretion, depending on

ARACHIDONIC ACID METABOLISM

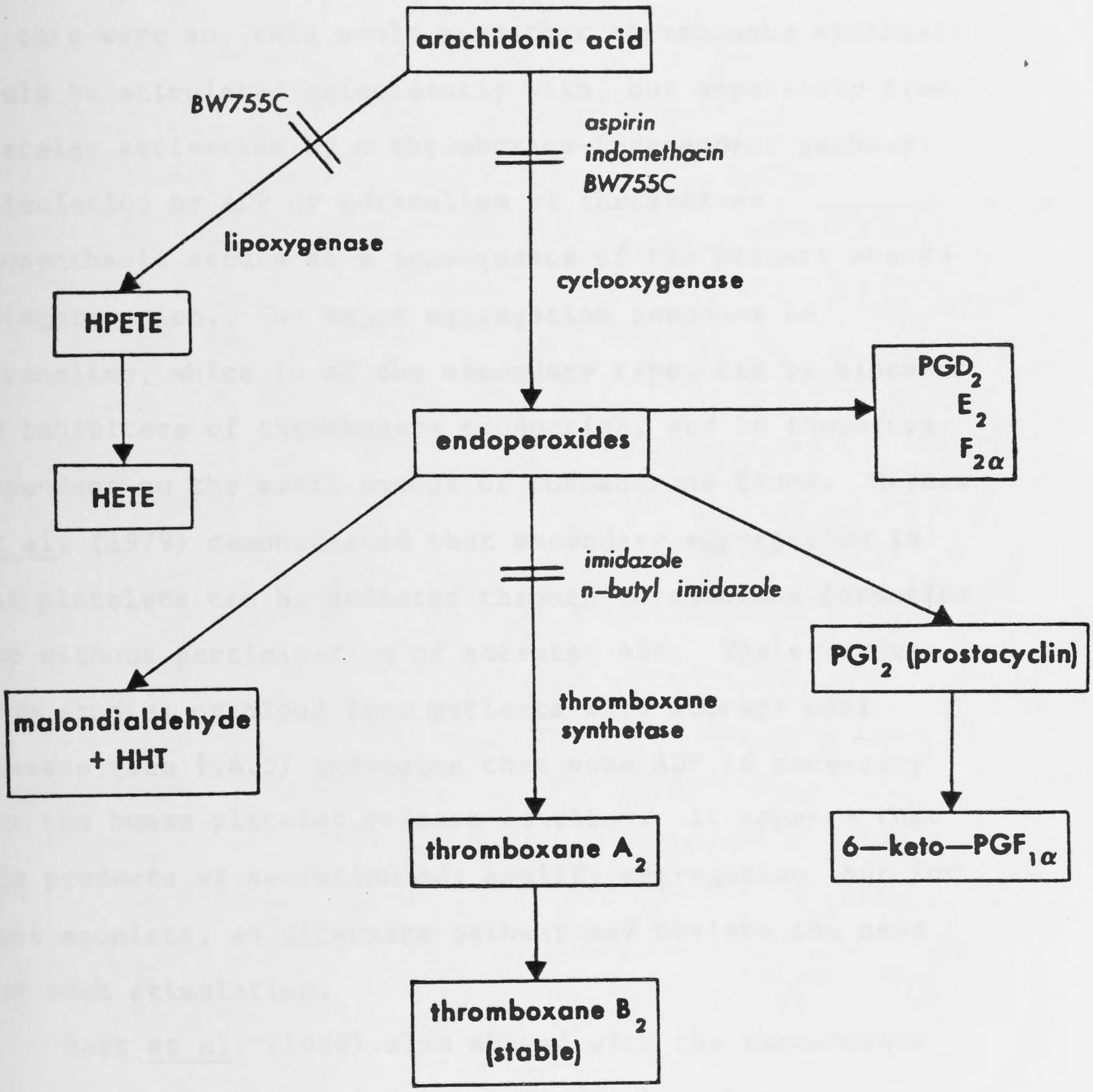

Figure 1.1 Arachidonic acid metabolism.

The combined activity of cyclooxygenase and thromboxane synthetase to produce endoperoxides and thromboxane A_2, a potent platelet aggregator is thought to be the most important pathway of arachidonic acid metabolism in platelets. The reactions are controlled by calcium and c'AMP: calcium (Ca^{2+}) promotes the formation of thromboxanes, while c'AMP has the reverse effect, largely through their respective effects on arachidonic acid release.
PG = prostaglandin HHT = heptadecatrienioc acid
HETE = 12L-hydroxy-5,8,10,14-eicosatetraenoic acid
≠ = inhibition.

the stimulus used. Thrombin and collagen each appear to activate platelets by two possibly independent mechanisms. If this were so, this would mean that thromboxane synthesis could be stimulated coincidently with, but separately from, platelet activation by a thromboxane-independent pathway. Stimulation by ADP or adrenaline of thromboxane biosynthesis occurs as a consequence of the primary events in aggregation. The major aggregation response to adrenaline, which is of the secondary type, can be blocked by inhibitors of thromboxane production, and is therefore dependent on the small amount of thromboxane found. Meyers et al. (1979) demonstrated that secondary aggregation in cat platelets can be mediated through thromboxane formation but without participation of secreted ADP. The evidence from studies on blood from patients with storage pool disease (see 1.4.5) indicates that some ADP is necessary for the human platelet release reaction. It appears that the products of secretion may amplify aggregation, but for some agonists, an alternate pathway may obviate the need for such stimulation.

Best et al. (1980) also showed with the thromboxane synthetase inhibitor, 1-N-butyl imidazole, that prostaglandin endoperoxides alone contribute little toward platelet activation. Contrary to this, Flower and Cardinal (1979) found 1-N-butyl imidazole only partially effective in blocking platelet aggregation, and suggested that endoperoxides have significant activity. The advantage of endoperoxide formation as an intermediary could be in the

facility for transformation to alternative prostaglandins, depending on the enzyme stimulated (Samuelsson, 1977). Endoperoxides have been found to inhibit PGE_1-induced increases in c'AMP but not as effectively as thromboxanes (Gorman et al., 1978). Through the use of inhibitors of thromboxane synthase, it appears that the effect of the endoperoxides on c'AMP levels is due to their conversion to TXA_2 (Gorman et al., 1977b).

Gerrard et al. (1978a;1978b) reported indirect evidence that endoperoxides and TXA_2 could act as intracellular calcium ionophores within platelets. Platelet activation induced by these prostaglandins is thought to be mediated by calcium mobilization from the dense tubular system (Gerrard and White, 1978) where thromboxane synthesis occurs (Gerrard et al., 1976). In this fashion, TXA_2 can act in direct opposition to the function of c'AMP which decreases calcium in the cytosol. Recently, however, Menashi et al.(1984) reported that neither thromboxanes, endoperoxides nor phosphatidic acid can act as ionophores.

Siess et al.(1983) suggested that endoperoxides or thromboxane A_2 are responsible for their finding that exogenous arachidonic acid stimulates the phospholipase C pathway. Formation of diacylglycerol and phosphatidic acid are important for platelet activation. Rittenhouse (1984) found also that phospholipase C activation by the ionophore A23187 was dependent on cyclooxygenase products and ADP.

Inhibition of platelet aggregation by prostaglandins

may also occur, without measurable increases in c'AMP levels, although this may represent the limit of discrimination of the assays. Sinha and Colman (1978) reported that PGE_1 induces an alteration or formation of an inhibitory macromolecule in plasma which can regulate platelet function independently of c'AMP. Rao et al. (1981a) used PGI_2 at levels which caused inhibition of arachidonate-induced aggregation without raising c'AMP levels. The inhibition was correctable with adrenaline, again independently of c'AMP. No inhibitors of adenylate cyclase were used to eliminate an effect of c'AMP. These workers were investigating the influence of catecholamines on prostaglandin receptors and concluded that stimulation of adrenergic receptors by adrenaline uncouples PG-receptor activity through close physical association of the two receptors. In experiments with washed platelets, where platelet receptors may be altered, small concentrations of PGI_2 were found to stimulate aggregation due to ADP and thrombin (Jorgensen et al., 1980). PG-receptor interaction is not a straightforward system leading to adenylate cyclase activation.

1.5.4 Phospholipid Turnover

Phospholipids were initially of interest as a source of arachidonic acid for thromboxane production but phospholipid breakdown has also been associated with platelet aggregation independently of arachidonic acid conversion (Lapetina et al., 1978). Arachidonic acid can be released from several phospholipid classes by the action

of phospholipase A_2, which cleaves the acyl group in the 2-position. Phosphatidylcholine (PC), phosphatidyl-ethanolamine (PE) and phosphatidylserine (PS) are the preferred substrates of phospholipase A_2 and are present in the platelet membrane (see 1.2.1) (Billah et al., 1980). Phosphatidylinositol (PI) is broken down specifically by phospholipase C to diacylglycerol (Rittenhouse-Simmons, 1979). By the action of a lipase, arachidonic acid can be released from diacylglycerol (Bell et al., 1979). Arachidonic acid release is not, however, necessary for platelet activation, and other products of membrane phospholipase activity could be important (Lapetina et al., 1979).

The action of thrombin on PI turnover in human platelets has been extensively studied. A recent report (Lapetina et al., 1981b) describes the conversion of PI to phosphatidic acid which cycles back to PI via four consecutive enzymes (Fig 1.2). Calcium controls this cycle, in that it promotes the degrading enzymes and inhibits resynthesis, resulting in an accumulation of phosphatidic acid. c'AMP increases the resynthesis resulting in a decrease in phosphatidic acid. Lapetina et al. (1981a) conclude that arachidonic acid release occurs via conversion of phosphatidic acid to lysophosphatidic acid. They do not see the action of a lipase on the diglyceride as important, but rather platelet diglyceride kinase is more active, yielding phosphatidic acid. Again these studies are largely concerned with arachidonic acid

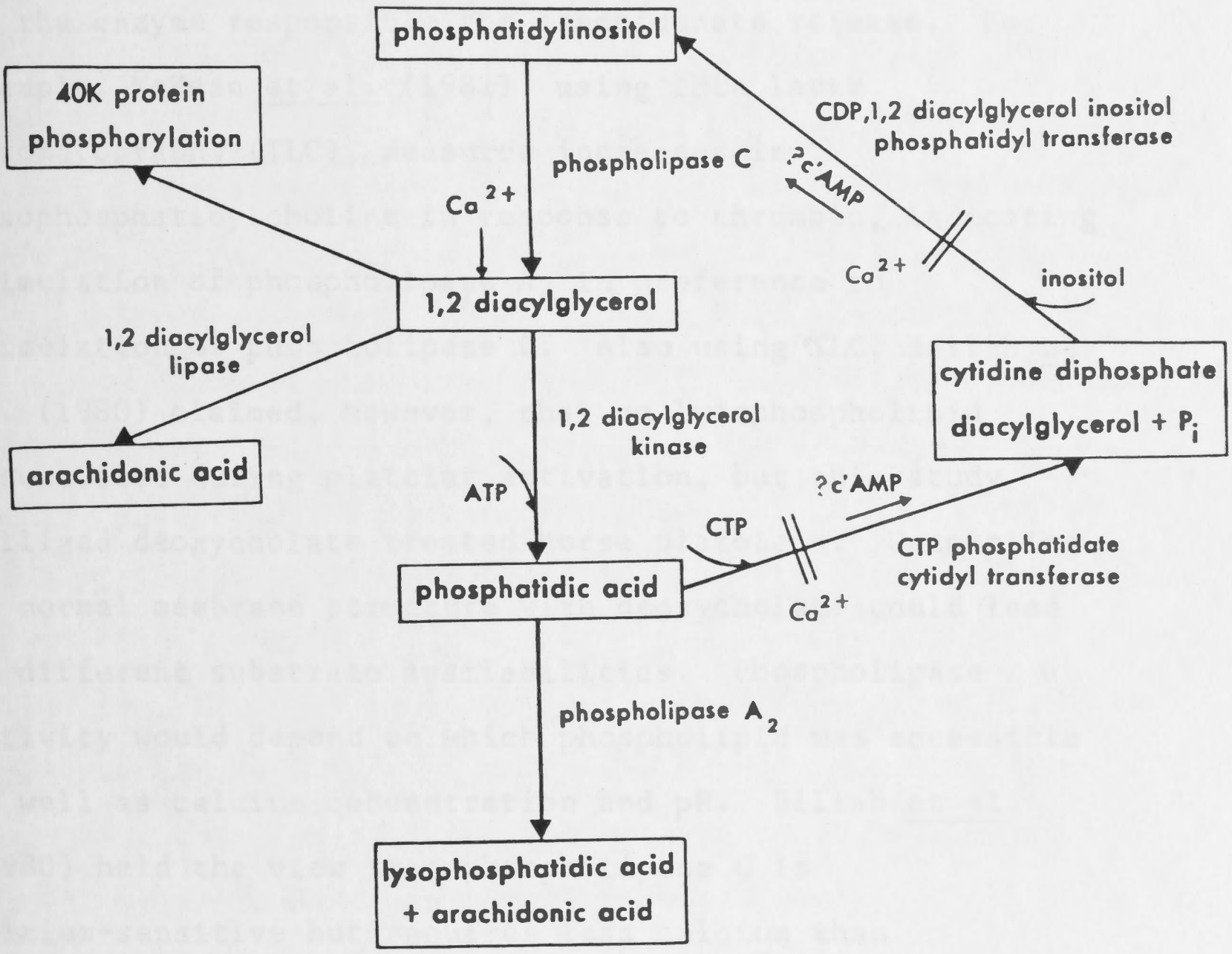

Figure 1.2 The phosphatidylinositol cycle.

This four enzyme cycle is controlled by Ca^{2+} and c'AMP. Increased Ca^{2+} levels favour the production of phosphatidic acid, while increases in local c'AMP favours the reconversion to phosphatidylinositol. Diacylglycerol occupies a significant position in being able to participate in three possible reactions.

release, rather than focussing on agents responsible for an initial and cyclooxygenase-dependent reaction within the platelet.

Much other work is concerned with the debate on the relative importance of phospholipase C or phospholipase A_2 as the enzyme responsible for arachidonate release. For example, McKean et al. (1981), using thin layer chromatography (TLC), measured increases in lysophosphatidylcholine in response to thrombin, indicating stimulation of phospholipase A_2 in preference to stimulation of phospholipase C. Also using TLC, Billah et al. (1980) claimed, however, that no lysophospholipid accumulated during platelet activation, but this study utilized deoxycholate treated horse platelets. Uncoupling of normal membrane structure with deoxycholate could lead to different substrate availabilities. Phospholipase activity would depend on which phospholipid was accessible as well as calcium concentration and pH. Billah et al. (1980) held the view that phospholipase C is calcium-sensitive but requires less calcium than phospholipase A_2 and could be stimulated immediately in the cytosol to act on exposed PI. Phosphatidic acid so produced could participate in the transfer of arachidonic acid to other phospholipids. There is also evidence for lysophosphatidic acids being able to act as calcium ionophores (Gerrard et al., 1979). Kannagi et al. (1980) published evidence supporting the idea that arachidonic acid needed to be mobilized within the

phospholipid classes during platelet aggregation. Rabbit platelets, after thrombin stimulation, exchanged arachidonic acid from the PE fraction to the PC fraction which is preferentially hydrolyzed. The idea of there being a store of arachidonic acid is attractive in terms of the benefits of control mechanisms for its release. Later work by Kannagi et al. (1981) modified their conclusions. They proposed domains of phospholipase-susceptible phospholipids, the mechanism of formation of which is unknown.

Thus far, phosphatidic acid has been postulated to act as a substrate itself for arachidonic acid release, but has not yet been given a role in prostaglandin-independent reactions. Considerable recent evidence indicates that diacylglycerol formed from PI may be a transmembrane signal for protein phosphorylation necessary for the release reaction (Kawahara et al., 1980). Diacylglycerol activates protein kinase C which is then capable of phosphorylating specific cellular proteins, especially those required for calcium independent reactions or those requiring very low levels of calcium (Naka et al., 1983). The action of activated protein kinase C, therefore, provides a possible mechanism for stimulation of release without an increase in resting cytosolic [Ca^{2+}].

Michell (1975) interprets a role for PI-turnover in opening cell-surface calcium-gates based on work with guinea pig ileum smooth muscle cells. The 'gate' is an ill-defined physiological system but implies control of calcium

availability, either from calcium uptake or release from membrane calcium-chelators. PI breakdown plays a role in the coupling between receptor-agonist interaction and the opening of the gates. It is of interest to note here, also, that α_1-receptor activation is thought to involve PI turnover and the release of bound intracellular calcium as well as the entry of extracellular calcium (Fain and Garcia-Sainz, 1980). This conclusion is based on examination of various tissues but not platelets which may not possess α_1-receptors (see Chapter 4). It would certainly be of interest to associate catecholamine activity and PI turnover even though this would be unusual.

There are three species of phosphoinositide found in cell membranes: phosphatidylinositol and two polyphosphoinositols. The actual levels of any of these lipids may be small but their interconversion enzymes may be quite active. Broekman (1983) observed that the thrombin-induced loss of phosphatidylinositol-4,5-bisphosphate in platelets coincides with a decline of membrane-found calcium as monitored by chlortetracycline fluorescence. Hydrolysis of phosphatidylinositol-4,5-bisphosphate yields inositol-1,4,5-trisphosphate which seems to act by mobilizing intracellular calcium (Berridge,1984).

A simple measurement of increased PI-turnover does not indicate which phospholipid products are formed, nor which would be responsible for specific activities within the platelet. A study of the action of one agonist, thrombin, does not allow for the possibility of alternate actions by other agonists on the platelet membrane. Billah and Lapetina (1982a) found that thrombin induces a rapid loss of phosphatidylinositol-4,5-bisphosphate but not phosphatidylinositol-4-monophosphate. This thrombin-stimulated loss is linked to receptor activation and is insensitive to calcium mobilization as shown by independence from calcium inhibitors (Billah and Lapetina, 1982b). In contrast to thrombin, activation by the ionophore, A23187, is totally dependent on the formation of cyclooxygenase products and ADP which form as a consequence of a receptor coupled event(Rittenhouse, 1984). The addition of A23187 to human platelets induces a transient drop in the amount of phosphatidylinositol-4,5-bisphosphate, a decrease in the amount of PI, and the formation of diacylglycerol and phosphatidic acid, producing lysophosphatidylinositol and free arachidonic acid.

The current understanding of the mechanism of activation of platelets via phosphatidylinositol turnover involves, therefore, the combined activities of two second messengers, diacylglycerol and inositol-1,4,5-

trisphosphate. Agonist receptor interaction induces the formation of these messengers from the hydrolysis of phosphatidylinositol-4,5-bisphosphate formed from part of the pool of PI (Berridge, 1984). Inositol-1,4,5-trisphosphate mobilizes calcium which then promotes protein phosphorylation via a calmodulin-dependent kinase. Diacylglycerol, in addition to forming phosphatidic acid and free arachidonic acid, may also be involved in calcium-independent phosphorylation via protein kinase C. Either protein phosphorylation may lead independently to the platelet physiological response of shape change and secretion, but a synergistic interaction between the two may be involved in the full physiological response(Kaibuchi et al., 1983).

1.5.5. Platelet Activating Factor

Platelet activating factor (PAF), is a glycerophospholipid with the proposed structure of 1-O-alkyl-2-acetyl-sn-glyceryl-3-phoshorylcholine (Demopoulos et al., 1979). PAF is released from various cells, including basophils, macrophages and platelets upon immune and non-immune challenge (Vargaftig et al., 1980a). It is one of the most potent aggregating substances, since concentrations as low as 1nM activate washed rabbit platelets. Plasma reduces its activity and human platelets are less sensitive.

Cazenave et al. (1979) studied the type of aggregation induced in rabbit platelets by PAF. They found the aggregation to be cyclooxygenase-independent and to occur

independently of serotonin release. PAF-induced aggregation can be inhibited by the prostaglandins, PGE_1 and PGI_2, possibly via c'AMP-induced reduction in available calcium ions. The strongest inhibitors of PAF-induced platelet aggregation are drugs that are known to be lipid soluble, and therefore membrane soluble. The mechanism of this inhibition is unknown and the drugs used are not specific.

PAF will induce aggregation in thrombin-degranulated platelets and in previously PAF-degranulated platelets(Cazenave et al.,1979). Thrombin can also activate PAF-degranulated platelets. A23187, thrombin and collagen cause release of PAF from rabbit platelets but ADP and arachidonic acid do not (Chignard et al., 1979; 1980). This indicates that PAF formation is not a general consequence of platelet activation but rather is part of a specifically induced pathway.

Kloprogge et al.(1983) found that the first wave of aggregation due to PAF is independent of cyclooxygenase products and ADP, but the second wave, accompanied by secretion was sensitive to cyclooxygenase inhibition and ADP removal. Despite several conflicting reports on the importance of platelet PAF, these authors suggest it plays only a minor role, if any, in aggregation.

Investigations of a mechanism for PAF induced aggregation have revealed that it stimulates PI turnover in human platelets (MacIntyre and Pollock, 1983), stimulates metabolism of phosphoinositides in horse platelets (Billah

and Lapetina, 1983) and that PAF promotes an early degradation of phosphatidylinositol-4,5-bisphosphate in rabbit platelets (Mauco et al., 1983).

1.6 AIMS OF THIS PROJECT IN RELATION TO PRESENT MODELS OF PLATELET AGGREGATION.

Mechanisms of platelet aggregation can be viewed in terms of pathways. A pathway is a series of events which lead to the same outcome in the platelet, such as aggregation or secretion. Since ADP can induce aggregation independently of thromboxane production and further release, ADP has been seen as having its own pathway for aggregation. Thromboxane mediated aggregation by collagen, thrombin, A23187 and adrenaline has been seen as another pathway. Since aggregation is still possible in the presence of both prostaglandin synthesis inhibitors and ADP degrading enzymes, a third pathway was proposed. Platelet activating factor was thought to be the mediator of this third pathway, but seems to have been excluded. Activation of protein kinase C by diacylglycerol may occur independently of both ADP and calcium. Formation of ADP and release of calcium augment the platelet response. Calcium may stimulate phosphorylation of contractile proteins and thromboxane formation which in turn may stimulate phospholipase C.

This thesis is concerned with examining further, various aspects of the enhancememt of human platelet responses by adrenaline. Adrenaline may participate in platelet activation as a potentiator for other agonists.

As mentioned (1.4.1), it is possible that the importance of adrenaline, *in vivo*, is in its sensitization of platelets to respond more effectively to other agents. Adrenaline needs to be considered when studying platelet reactivity, since psychological and other forms of stress may be risk factors for coronary artery disease, and can produce raised levels of this catecholamine. For example, even in the mildly stressful situation of public speaking, adrenaline levels have been reported to rise 3-fold (Dimsdale and Moss, 1980).

The mechanism of adrenaline potentiation has been investigated by others but not yet clarified. Although adrenaline-induced secondary aggregation depends on the cyclooxygenase pathway of arachidonic acid metabolism, adrenaline potentiation of ADP- and arachidonate-induced aggregation is not prevented by cyclooxygenase inhibitors (Rao *et al.*, 1980a) and neither is the first phase. It has been suggested, particularly with the initial stimulation by ADP,that adrenaline increases calcium uptake from plasma (Owen and Le Breton, 1980), that adrenaline releases membrane-associated calcium, or that it directly promotes deacylation of phospholipids (Rao *et al.*, 1980b). There is, however, no evidence that adrenaline increases intracellular calcium prior to secretion, although Brass and Shattil(1982) found that adrenaline can increase the amount of calcium bound to the platelet surface membrane. With the use of the chlortetracyline fluorescent probe, Owen and Le Breton(1981) found that adrenaline induced

significant mobilization of intracellular calcium but this was reduced in the presence of EDTA. Adrenergic involvement in phospholipid turnover has been reported in other tissues (Abdel-Latif, 1974; Michell, 1975; Fain and Garcia-Sainz, 1980). The mechanism of adrenaline potentiation warrants closer study. In particular, the phospholipids of membranes which could lead to calcium ion mobilization or redistribution across membranes, independently of thromboxane synthesis are of interest in relation to adrenergic interaction.

No one has been able to associate phospholipid changes with the initiating events of adrenaline activation whereas changes have been associated with the later event, secretion (Deykin and Snyder, 1973). Adrenergic effects on phospholipid turnover are usually associated with α_1-receptor activation(Fain and Garcia-Sainz, 1980). α_1-receptors have been reported to be present on human platelets(Grant and Scrutton, 1979) but only with a frequency of 20%(Scrutton, 1981). α_1-receptors are less effective in inducing aggregation than are α_2-receptors(Grant and Scrutton, 1979), the α_2-receptor being the primary mediator of the proaggregatory response to adrenaline (Grant and Scrutton, 1980b).

The expected response following α_2-receptor interaction is inhibition of adenylate cyclase(Fain and Garcia-Sainz, 1980). Primary aggregation is dependent on stimulus-response coupling following α_2-receptor activation by adrenaline(Grant and Scrutton, 1979) but the events of

this coupling are not precisely known. No one has been able to show that adrenaline inhibits adenylate cyclase in resting, intact platelets.

ADP-induced aggregation can be potentiated by subthreshold concentrations of several agonists, including adrenaline, and this is examined in Chapter 3. ADP-induced aggregation provides a thromboxane-independent system when cyclooxygenase or thromboxane synthetase inhibitors are used. ADP does not release PAF (Chignard et al., 1980), but does cause an increase in membrane phosphatidic acid (Lloyd et al., 1972; Lapetina and Cuatrecasas, 1979). The possibility of α-adrenergic activity also leading to increased phospholipid turnover suggests a mechanism for adrenaline potentiation of ADP-induced aggregation. Adrenaline potentiation of ADP-induced aggregation was therefore studied in the presence of various inhibitors.

Cyclooxygenase inhibitors are the subject of Chapter 4. Adrenaline potentiation without arachidonic acid metabolism is compared with adrenaline potentiation when arachidonic acid metabolism is stimulated, both subsequent to ADP-activation and as a result of exogenous arachidonic acid. This aspect of the project highlights the involvement of adrenaline in non-thromboxane initiated events under conditions of potentiation, even though the usual response to threshold levels of adrenaline results in thromboxane-dependent aggregation and release. An attempt was also made to examine adrenaline potentiation at stages

prior to cyclooxygenase activation by blocking post-receptor events with different types of inhibitors.

In Chapter 5 a study is reported of the use of putative inhibitors of phospholipase A_2 which inhibit the release of arachidonic acid. The aim was to observe aggregation in the absence of phospholipid changes arising from the action of phospholipase A_2. The results suggest that adrenaline affects the phospholipase C pathway of phosphatidylinositol turnover. To explore this further, the direct effects of these inhibitors on exogenous phospholipase C were studied and compared with their effects on adrenaline potentiation (Chapter 6).

The first studies were based on observations of aggregation, release of ATP and thromboxane B_2 production. To answer questions on associated phospholipid turnover, phospholipid analysis by Whatman SG-81 paper chromatography was undertaken (Chapter 7). With this method, preliminary observations of phospholipid metabolism were made in relation to α-receptor activation.

With these combined studies a model for the mechanism of adrenaline potentiation has been examined. This model includes the interaction of adrenaline with α-receptors, and subsequent events at the membrane level involving phospholipid turnover, which lead to protein phosphorylation and calcium movement. Subsequently, thromboxane synthesis occurs and serves to amplify the original signal.

CHAPTER 2

METHODS AND MATERIALS

2.1 METHODS

2.1.1. Aggregation Studies

2.1.1.1 Advantages and Disadvantages of Studying Platelet Aggregation *in vitro*.

Platelet aggregation studies are performed *in vitro* since it is much easier to obtain a blood sample from a human donor than it is to examine blood and vascular tissue, interactions *in situ*. Setting up a system in the laboratory enables parameters to be defined and standardized. Several different environmental factors can be controlled simultaneously. There is the additional advantage that potentially toxic substances can be used *in vitro*.

There are, of course, several disadvantages in attempting to simulate the physiological environment. Platelets studied in isolation, are not influenced by vascular tissue which is multifactorial. Anticoagulants have to be used in the preparation of platelets from blood. These compounds very likely affect the reactivity of the platelets. Platelet responsivness alters with time once blood is removed from the circulation. Blood flow dynamics are reproduced by a simple procedure only, that of stirring magnetically.

The physical handling of platelets gives rise to major problems, particularly if washed platelets are required. Release of granular products, if not actual aggregation, occurs. Extraordinary lengths have to be taken to ensure

no release of PF_4 and βTG and other products from the α-granules. The washing process may also remove a necessary platelet atmosphere containing fibrinogen, thrombin and calcium. All conclusions based on *in vitro* studies should therefore be made with an awareness of these differences from the *in vivo* situation.

2.1.1.2 Platelet Preparation in Citrated Blood Versus Heparinized Blood.

The choice of anticoagulant best suited to aggregation studies of platelets has been a matter of controversy for some years. It has been claimed that the release reaction induced by ADP is an artifact in citrated plasma brought about by the reduction in available calcium but is independent of such a reduction in magnesium (Mustard *et al.*, 1975; Macfarlane *et al.*, 1975). According to their reports platelets in plasma which is anticoagulated by heparin or hirudin, or platelets washed and suspended in a medium which contains 1-2mM Ca^{2+} do not undergo the release reaction when they are aggregated by ADP. The concentration of calcium in the circulation is about 1mM (Hansen and Theodorsen, 1971), but the optimum concentration of calcium for aggregation is below this (Heptinstall, 1976).

EGTA (ethylene-glycol-bis (β-amino-ethyl ether) N,N'-tetra-acetic acid) behaves in the same manner as citrate in its effect on aggregation due to ADP, confirming that a reduction in calcium ions promotes the release reaction (Huzoor-Akbar and Ardlie, 1978). A direct effect of

citrate is therefore not the cause of secondary aggregation. ADP-induced biphasic platelet aggregation will occur in the absence of citrate. Huzoor-Akbar and Ardlie (1976; 1977; 1978) proposed that thrombin is necessary for the release reaction which would explain the inhibitory effect of heparin and hirudin.

Recently a detailed comparison was made of the reactivity of platelets in plasma anticoagulated with citrate, heparin or hirudin (Lages and Weiss, 1981). Variable responses between different donors were observed for heparinized platelet-rich plasma, with respect to secretion, but not the extent of aggregation by ADP. Adrenaline-induced aggregation showed variability in both secretion and extent of aggregation in heparin compared to citrate. Hirudin consistently prevented the release reaction which supports earlier evidence that thrombin is involved in platelet reactions caused by ADP, adrenaline and collagen (Ardlie and Han, 1974; Huzoor-Akbar and Ardlie, 1976; 1978).

Hwang and Le Blanc (1981) proposed that heparin has aspirin-like activity in that it inhibits formation of endoperoxide metabolites. If this were so, lack of thromboxane formation and secondary aggregation could be an artifact of heparinized plasma. Heparin, added to citrated plasma inhibited thromboxane production. Collagen stimulation of thromboxane production was reduced in heparinized plasma compared to citrated plasma.

Heparin itself is reputedly an aggregating agent in some circumstances but the effects are variable and inconsistent (Wissler and Gitel, 1976). Heparin was shown to induce aggregation at 4°C and at 37°C of washed platelets derived from patients exposed to severe stress (Mims et al., 1977). Other authors claimed that storage at 37°C protects the platelets from aggregation due to heparin (Han and Ardlie, 1974). Heparin has also been reported to potentiate the platelet response to ADP (Thomson et al., 1973). Lages and Weiss (1981) could induce aggregation with 2.5U/ml to 100U/ml heparin.

Reches et al. (1979) showed that heparin (0.4-1.62U/ml) inhibits by 50%, PGE_1-sensitive adenylate cyclase and antagonizes PGE_1 antiaggregating effects on platelets. These authors claim that their observations demonstrate that heparin induces a secondary phase of aggregation but neither release of serotonin, nor ATP, nor thromboxane production was investigated in their study. Their definition of secondary phase seems to depend only on the lack of reversal of aggregation.

Certainly, the anticoagulant has to be specified to enable any interpretation to be made of an aggregation study. For one of the studies in this thesis, low concentrations of heparin were chosen in order to more closely reflect the situation in circulating blood. This practice incurred several difficulties, including insufficient anticoagulation of the blood from some donors.

For other aggregation studies, where maximal platelet sensitivity was not required, and a stable system was desired for comparisons to be made between experiments, citrate was used as the anticoagulant. Although trisodium citrate (10mM) chelates calcium ions, it does not, unlike EGTA, leach calcium out of the platelets.

2.1.1.3 Preparation of Platelet-Rich and Platelet-Poor Plasma from Fresh Whole Blood.

Blood donors were healthy human volunteers who had not taken any aspirin-containing drugs in the previous fortnight. Donors taking steroids, antihypertensive drugs or antiasthmatic drugs were also avoided.

Anticoagulant was mixed gently with the blood which was then contrifuged at 100g for 15 mins. The platelet-rich plasma (PRP) was aspirated off, avoiding the layer near the white cells, using a siliconized-glass Pasteur pipette. This plasma was pooled in a polypropylene vessel, then taken up in a syringe from which air was subsequently expelled. The syringe was tightly capped and placed in a 37°C water bath for the duration of the experiment. Under these storage conditions the aggregation response remains constant for 2.5-3 hours. The platelets were not used during the first ten minutes of storage. Platelet counts were determined using a Coulter Counter, Model ZF.

A sample of PRP was spun in an Eppendorf microcentrifuge at 8000g for 2 mins. to sediment the platelets. The platelet-poor plasma (PPP) so formed was

then used to establish 100% light transmission using an aggregometer.

2.1.1.4 Preparation of Washed Platelets.

Method A. PRP was prepared from blood anticoagulated with acid citrate. The PRP was centrifuged at 1100g for 15min to form a platelet pellet. The plasma was poured off, the tube allowed to drain and washing fluid (pH6.5) was added. The platelets were resuspended in this washing fluid by pipetting up and down and a further 2ml of acid-citrate dextrose was added per 8ml fluid prior to further centrifugation. The platelets were resuspended in suspending fluid (pH7.4)(see below) and stored in an air-tight syringe at 37°C.

Method B. PRP was obtained from the Red Cross Blood Bank, derived from a unit of blood. The PRP was spun at 100g in the presence of 2ng/ml prostacyclin (PGI_2) to remove more red blood cells. The supernatant PRP was then centrifuged with 300ng PGI_2/ml at 1100g for 15min to form a platelet pellet. These platelets were resuspended in washing fluid (pH7.4) with 150ng PGI_2/ml and apyrase (1ml/50ml plasma)(see below for preparation of apyrase). The platelets were again centrifuged at1100g for 15min and the resultant pellet resuspended in suspending fluid (pH7.4). The platelets were stored in an airtight syringe at 37°C. A recovery time of 30min was allowed to reverse the effects of the PGI_2. This method is a modification of Vargas et al.(1982).

2.1.1.5 Observation of Aggregation using an Aggregometer and Lumi-aggregometer.

A turbidometric method for observation of platelet aggregation was devised by Born in 1962. This widely accepted technique was adopted, using a Payton dual channel Aggregation Module to measure changes in light transmission through stirred samples of PRP in siliconized glass cuvettes.

Changes in light transmission were recorded with a Riken-Denshi SP-H6P recorder with a paper speed of 600mm/hour. A baseline was established for unstimulated swirling platelet-rich plasma (zero transmission) and 100% transmission was set for PPP. This system allowed for observation of shape change when the turbidity increased slightly and subsequent aggregation associated with an increase in light transmission.

A Chrono-Log Lumi-Aggregometer was used to measure the release of ATP associated with platelet aggregation Firefly lantern extracts were prepared by homogenizing 30mg Sigma FFT in 1ml of 100mM HCl, filtering through gauze and finally centrifuging at 1,000g for 5 mins. 25µl of this preparation was added to 500µl PRP containing 20µl of 154mM $MgCl_2$.

Standard solutions of ATP were prepared freshly on the day of the experiment in Reagent Diluent (RD, see Section 2.2). One test for each of a range of concentrations of ATP was used to determine the number of divisions of

recorder deflection for that concentration. In this manner the ATP assay was semi-quantitative.

2.1.2 Radioimmunoassay of Thromboxane B_2

2.1.2.1 Antibody Binding and Crossreactivity

TXB_2 antiserum (#280 D21) was a kind gift from Dr L. Levine, Waltham, Mass. USA. With the number of counts of the tracer set at 4,000 cpm per 100µl, 50% binding was achieved with a final dilution of antiserum of 1 in 20,000. A dilution of 1 in 10,000 shifted the standard curve to the right for no increase in binding. As binding displacement was required for standards in the lower range and, of course for conservation of antibody, the 1 in 20,000 dilution was chosen. There was less than 1% cross-reactivity with $PGF_1\alpha$ and $PGF_2\alpha$.

2.1.2.2. ^3H-TXB_2 Characteristics

The radioactive tracer, ^3H-TXB_2, was obtained from New England Nuclear in different lots which varied in radiochemical purity, as assessed by thin layer chromatography, between 98 and 98.5%. Each lot had a different specific activity but was counted prior to use and diluted to achieve a final dilution of approximately 4,000 cpm per incubation tube.

2.1.2.3 The Assay

In polystyrene, Wasserman tubes, the radioimmunoassay was set up with each incubate consisting of 100µl each of sample or standard antibody and tracer, added in that order. The tubes were incubated at 4°C for at least 18 hrs and no longer than 2 days. An intra-assay control

consisted of placing standard TXB_2 at various positions throughout the one assay set of tubes. Inter-assay variability was controlled by placing in each assay a standard set of tubes from a batch kept frozen.

The standard curve was formed with the dilution from a 2µg aliquot to make a series of 8 standards from 8pg to 1000pg/100µl. The samples assayed contained plasma which was found to interfere with antibody binding so each incubate contained 10µl of sample only. Consequently each standard TXB_2 tube had 10µl PRP (inactivated with 100µM indomethacin) added. Each tube was vortexed and then incubated.

2.1.2.4 Separation, Counting and Calculations

Separation of bound from unbound tracer was achieved by the addition of 300µl of dextran-coated charcoal. The mixture of charcoal, dextran and albumin was stirred for 10 min. on ice and the aliquot was rapidly added to each tube which was immediately placed in a centrifuge carrier and shaken.

5 mins after the addition of charcoal to the last tube centrifugation for 10 mins at 1500g on a MSE Coolspin was commenced. The supernatant fluid was decanted into a scintillation vial containing 5ml of Xylene/TritonX100 scintillant. The vials were capped, shaken vigourously and counted in a Packard scintillation counter.

Raw counts were converted to percentage binding by the formula $\%\text{Bound} = \frac{\text{Counts - NSB}}{\text{Total Counts}}$

NSB = non-specific binding, that is, the counts remaining in the supernatant fluid without the presence of antibody. A standard curve of percentage bound radioactivity versus amount of TXB_2 (pg) was plotted on semi-logarithmic paper. The unknown samples were then read from this curve and concentrations in pg/ml were converted to pmole/10^7 platelets for each experiment.

2.1.3 Phospholipid Analysis

2.1.3.1 Extraction of Membrane Lipids

Platelet reactions in a volume of 900µl were stopped by the addition of 1.25ml CH_3OH:10M HCl(50:1). The cuvette was rinsed out with 3.1ml $CHCl_3OH$ (25:32) and added to reaction mixture. This was partioned using 1.4ml $CHCl_3$ and 1.4ml water which was mixed and the mixture centrifuged at 1000g for 10min. The lower layer was dried under nitrogen at 45°C. The phospholipids were redisolved in $CHCl_3$:CH_3OH:10M HCl (200:100:0.1) containing 0.05% butylated hydroxytoluene (BHT) and stored at -20°C prior to chromatographing. This method is modified from Bligh and Dyer (1959) as used by Lloyd et al.(1972).

2.1.3.2 Paper Chromatography

The lipid extract was separated into different polar lipids in a two dimensional system on Whatman SG-81 chromatography paper. Two different solvent systems were tried with the second one being adopted for experiments. Both were taken from Wuthier (1976).

System A.

1st dimension (long) chloroform: diisobutylketone: methanol: acetic acid: water (45:30:15:20:4)

2nd dimension (short) chloroform: diisobutylketone: pyridine: methanol: water (30:25:35:25:8).

System B.

1st dimension (short) chloroform: methanol: 5M NH_4OH (64:34:4).

2nd dimension (long) chloroform: diisobutylketone: pyridine: methanol: water: acetic acid: formic acid (23:20:25:20:3:8:1).

46x57cm sheets of paper were cut into 9 equal-sized sheets 15.3x19.0cm and stored in a dessicator. Diisobutylketone was vacuum distilled prior to use. Solvent mixtures were prepared fresh daily and 100ml added to each tank at least 10min prior to introduction of the paper. TLC tanks were used into which four rolled cylinders of paper could stand side by side.

A spot of lipid extract was made in the lower left hand corner, applying 20-30µg P in about 20µl. The spot was made in the form of a streak about 3x7mm in the direction of the first run. The spot and labels were marked with a pencil. The paper was rolled and hand-sewn with a whip-stitch to form a cylinder. The paper was then placed in the chromatography tank after no longer than fifteen minutes.

The solvents were allowed to run to about 1cm from the top which took 2 hr for the first dimension (short) and 3 hr for the 2nd dimension (long). The paper was dried for 30min between the two runs by hanging in a fume cupboard. The cylinder was then cut open and resewn at right angles to the first.

After the second run, the paper was thoroughly dried again in the fume cupboard and prepared for staining which was done in a metal tray. The chromatogram was washed with distilled water and rinsed with 0.5M acetic acid. The staining was then achieved by soaking in rhodamine 6G (0.0012%) for 10min with gently rocking to ensure uniform contact.

The stained chromatogram was viewed under UV while still wet and the spots marked with a dull pencil. When the chromatogram had dried, it was again viewed under UV and the spots checked for colour changes. Lysolipids were detected by a change from pale yellow to blue on drying.

2.1.3.3 Phospholipid Estimation by Phosphorus Analysis. (Rouser et al., 1966)

Each spot was cut out, weighed and placed in a 15x1cm pyrex test tube. To each tube was added 750µl 70% perchloric acid containing 1ml/litre of 10% ammonium molybdate. The tubes were heated in a radiant heat apparatus at 180-200°C until the colour disappeared. To the cooled tubes 3ml of water was added and mixed. Next, 0.5ml 2.5% ammonium molybdate then 0.5ml 10% ascorbic acid was added to each tube, mixing between additions. The

tubes were incubated at 56°C for 30min. The silica was removed by centrifugation and the supernatant fluid read on a Beckman DU-8 spectrophotometer. A zero standard was used as a blank for the instrument. A standard curve was prepared using standard inorganic phosphorus solutions in the range 0.1 to 10µg/ml. Each unknown phosphorus was estimated from the standard curve allowing for the phosphorus content of the paper itself. The paper phosphorus was calculated from non-stained areas of the chromatogram which were weighed and digested as were the lipid spots. The estimation of phosphorus per mg paper allowed an estimation of phosphorus due to the paper alone from the weight of the spot.

true µgP/spot = (totalP/spot)-(mgpaper/spot X µgP/mg paper in blank areas)

2.1.3.4 Phospholipid Turnover as Indicated by ^{3}H-arachidonic Acid Incorporation

^{3}H-arachidonic acid was incorporated in the membranes of platelets by incubating at 37°C for 1 hr with 5µl ^{3}H-arachidonic acid (Amersham), specific activity 137Ci/m mol per 100ml of original PRP volume. (5µl=5µCi). The incubation was done in washing fluid, ensuring prostacyclin 150µg/ml was added for the incubation and again immediately prior to centrifugation. The platelets were then resuspended as usual and allowed to recover for 15min at 37°C.

Platelet reactions, extraction of lipids and chromatography were carried out as described above. The

spots were cut out and placed in scintillation vials to which 5ml of scintillant was added. The vials were counted in a Packard Tri-carb 460 scintillation counter which recorded counts as disintegrations per min. Different quenching due to the different sizes of stained paper was found to be insignificant.

2.2 MATERIALS

2.2.1 Buffers and Solutions.

Reagent Diluent (RD) is a modified Tyrode's solution with the following composition:8.0g/1 NaCl, 0.2g/1 KCl., 1.0g/1 $NaHCO_3$, 0.05g/1 NaH_2PO_4 at pH 7.4. This solution was prepared weekly and stored at 4°C. It was used to dilute reagents used in aggregation studies.

Washing fluid was prepared on the day required to give a final composition of: NaCl 8.0g/1,KCl 0.2g/1,$NaHCO_3$ 1.0g/1,NaH_2PO_4 0.05g/1, albumin 3.5g/1, glucose1g/1; pH7.4 or 6.5 at 37^{0}C. Apyrase was added (Ardlie et al., 1970; 1971) immediately prior to addition to platelets or 2ml extra ACD was added per 8ml fluid. The solutions were kept at 37°C prior to use. ACD = acid citrate dextrose which consists of 25g trisodium citrate, 14g citric acid and 20g dextrose per litre.

Suspending fluid was prepared on the day required and differed from the washing fluid in containing, in addition, $MgCl_2$ 0.009g/1 and $CaCl_2$ 0.022g/1;pH7.4,37^{0}C.

Stock fibrinogen for adding to washed platelets in order that adrenaline and ADP may cause aggregation. It

was prepared from Kabi human fibrinogen, 1g was dissolved in 20ml normal saline, and placed on gentle agitation. 2ml of 20% $Al(OH)_3$ was added when fibrinogen dissolved. The solution was left for 10min at room temperature for adsorption and centrifuged at 2000g for 10min. The adsorption was repeated with another 2ml of 20% $Al(OH)_3$. The fibrinogen solution was then dialyzed against normal saline at room temperature with four changes overnight. The dialyzed solution was ultracentrifuged at 37,000g for 20min ambient temperature on a Beckman J2-21 centrifuge. The protein concentration was estimated by a modified Lowry method (Peterson, 1977). Aliquots of 100µl were stored at -20°C and thawed on the day required.

The stock solution was estimated to be 1.2g%. An aliquot was thawed by warming and used at 20µl per 750µl platelet suspension. This gave a final concentration of approximately 0.3mg/ml.

Apyrase was prepared from potatoes and stored in 1ml aliquots at -20°C. Peeled, sliced potatoes were homogenized in a Waring blender (10kg/litre d.H_2O). The suspension was stirred a further 30min at room temperature and then pressed through 6 layers of cheese cloth. The effluent was centrifuged at 900g R.T. for 10min. The supernatant fluid was refiltered and centrifuged. Further steps were carried out at 0-10°C. The supernatant fluid was brought to 25mM $CaCl_2$ and stirred 15min at 4°C. The solution was allowed to settle for 1 hr at 4°C, then

centrifuged 3500g for 20min at 4°C. The precipitate was resuspended in 1.0M $CaCl_2$, stirred 60min 0-4°C and centrifuged as before. The supernatant fluid was then dialyzed against 20l 0.1M KCl for 24hr 4°C. The supernatant fluid was combined with saturated ammonium sulphate and stirred for 40min, then again centrifuged. The precipitate was redissolved in 25ml distilled water and dialyzed twice against normal saline overnight.

For platelet counting, 5µl of platelets were added to 15 mls of Isoton II (azide free) balanced electrolyte solution.

TRIS-gel buffer for radioiminunoassay was a 15mM TRIS-base (Sigma) solution with 500mg/l gelatin (Ajax) added. A stock solution of 150mM TRIS-HCl, pH 7.4 was kept at 4°C. As required this was diluted one-tenth, gelatine dissolved in it by warming, then adjusted to pH 7.4 at 4^0C.

2.2.2 Aggregating Agents

Adenosine diphosphate (ADP)(Sigma) was made up in 100µl aliquots of 10^{-2}M and stored at -20°C. Just prior to an experiment this solution was further diluted with RD.

Adrenaline bitartrate (Calbiochem San Diego, CA, USA) was prepared and stored the same way as ADP, except that adrenaline was kept in the dark.

Arachidonic acid was converted to the potassium salt by dissolving 100mg in 11.25ml ethanol containing 18.42mg KOH. This was stored in 1ml aliquots in the dark at -20°C. Immediately prior to each experiment, the potassium

arachidonate was dried down under nitrogen and redissovled in RD.

5 hydroxytryptamine (serotonin) from Sigma was prepared as required.

A 20U/ml ampoule of Vasopressin (Pitressin(R), Parke-Davis) was opened for each experiment and diluted as required in RD.

Noradrenaline (Sigma) was prepared and stored the same way as adrenaline.

Clonidine (Catapres pure substance) a gift from Boehringer Ingelheim, was prepared and stored the same way as adrenaline.

Phenylephrine-HCl(Sigma) was kept dry at 4°C and prepared as required.

Phospholipase C (Sigma) of specific activity 20U/mg protein or 8U/mg solid was stored at -20°C and prepared in RD as required.

Thrombin (Parke Davis, bovine origin) was diluted in RD to 1000U/mland stored in 100µl aliquots at -20°C.

2.2.3 Inhibitors

Acetysalicylic acid, aspirin (ASA) (BDH,general purpose reagent) 50mM was made freshly each day with 90mg per 10ml. It was dissolved by stirring and adding NaOH until the pH was approx. 7.3. This solution was diluted as required and used at one-tenth volume of PRP.

Indomethacin (Sigma) was prepared in absolute ethanol at a concentration of 10mM to be used at one-hundredth the volume of PRP at a final concentration of 100µM.

BW755C; 3-Amino-1-[M-(trifluoromethyl)-phenyl]-2-pyrazoline, a gift from Burroughs-Wellcome, was dissolved at a concentration of 100µg/ml (380µM) in RD and used at one-tenth volume in PRP.

n-butyl-imidazole (Koch-Light), a liquid stored at 4°C was freshly diluted in RD to obtain a concentration of 50mM and was used at one-tenth volume in PRP. p-Bromophenacyl bromide, BPB (Koch-Light) was made freshly in dimethylsulphoxide (DMSO) at a concentration of 5mM to be used at one-hundredth volume in PRP.

Tosylphenylalaninechloromethylketone (TPCK) (Sigma) was also dissolved in DMSO.

Dibenamine and phenoxybenzamine were both gifts from Smith, Kline and French, Frenchs Forrest, NSW. Both were prepared freshly in ethanol and added at one-hundredth volume or diluted further in saline.

Hirudin (Sigma Chemical Co.) was dissolved freshly in RD.

Isoptin (verapamil)(Knoll, 2.5/ml ampoules) was stored at 4°C and diluted in RD as required.

Largactil (chlorpromazine)(May and Baker, 25mg/ml ampoules) was stored at 4°C and diluted as required. This preparation also contained sodium sulphite, sodium metabisulphite and sodium citrate.

Phenergan (promethazine)(May and Baker, 25mg/ml ampoules) was stored at 4°C and diluted as required as was trifluoperazine.

Mepacrine(quinacrine)(May and Baker) was prepared freshly in DMSO.

Prostacyclin, a gift from the Upjohn Company, Kalamazoo, Michigan, U.S.A. was prepared in 0.05M TRIS buffer, pH9.3 freshly each week.

Trimetoquinol (S-TMQ)was a kind gift from Yoshio Iwasawa, Tanabe Sekako Co.Ltd.Japan. and was stored at -20°C dissolved in DMSO as required

Spermine (N,N-bis[3-aminopropyl]-1,4 butanediamine) Sigma was dissolved in RD.

2.2.4 Anticoagulants.

Citrate, (trisodium salt) was kept as a stock solution of 100mM and used in a ratio of 1ml to 9ml of blood, giving a final concentration of 10mM.

Heparin (Injection B.P., Allen and Hanburys) was used freshly from an unopened ampoule, diluted in RD to give desired concentration from a one-in-ten dilution. Dried sodium heparin (mucous) from Commonwealth Serum Laboratories was dissolved freshly as required. Heparin from Evans Medical Ltd., Liverpool, England was used for some experiments.

Acid citrate dextrose (ACD) was used as anticoagulant for some preparations of washed platelets. It was prepared from 25g trisodium citrate, 14g citric acid and 20g dextrose per litre.

2.2.5 RIA Reagents

Antibody against thromboxane B_2#280 D21 courtesy Dr T. Levine, Waltham, Mass. U.S.A. was divided into 5µl aliquots

which were freeze dried. As required, each aliquot was reconstituted to 1ml with TRIS-gel buffer pH 7.4 and diluted on the day of assay. Anything remaining of the reconstituted aliquot was stored at 4°C, not frozen, and discarded after one week if not used.

^{3}H-TXB_2 (New England Nuclear) was stored at -20°C with the addition of 5ml of ethanol. On the day of assay the appropriate volume was removed and diluted with TRIS-gel buffer pH 7.4.

Dried TXB_2 (U51541) for use as a standard was a gift from The Upjohn Companay, Kalamazoo, Michigan, USA. 0.569mg was weighed on a Perkin-Elmer Autobalance AD-27, weighed by difference. This sample was then dissolved in 50ml water in a standard flask from which 200 aliquots were freeze dried and kept at -20°C.

Dextran coated charcoal was prepared freshly by combining 10mg dextran T-10, 10mg bovine serum albumin and 60mg charcoal (Norrit A) per 10ml TRIS-gel at 4°C.

2.2.6 Solvents for Paper Chromatography

The solvents used for the paper chromatograph were analytical reagents, except for diisobutylketone (2,6-dimethyl-4-heptanone, Ega-Chemie) which was redistilled by vacuum distillation. The other solvents were chloroform AR (Ajax), methanol AR (Ajax), pyridine (Ajax), 5N ammonia (NH_4OH)(Commonwealth Ammonia Co.), acetic acid (Ajax) formic acid (BDH) and nanopure distilled water.

2.2.7 Reagents for Phosphorus Assay

Digestions were performed in 70% perchloric acid (G.

Frederick Smith Co., Columbus, Ohio). Colour development in the assay was produced by ammonium molybdate (Baker Chemical Co.) and ascorbic acid (Calbiochem). Nanopure distilled water was used for all solutions.

CHAPTER 3

MULTIPLE AGONISTS *IN VITRO*

3.0 SUMMARY

This study examined the interaction between several agonists of human platelet aggregation in blood collected into low concentrations of heparin, in an attempt to reproduce the *in vivo* situation. These conditions were chosen because multiple agonists are present in circulating blood. Another characteristic of the haemostatic mechanism may be the physiologic regulation of blood coagulation by vascular endothelial heparan sulphate. Irreversible aggregation was observed *in vitro* at pathological levels of adrenaline, when used in combination with ADP and vasopressin. Even lower levels, in the physiological range, caused aggregation when four agonists were combined. *In vivo* aggregation might therefore be expected to occur under certain circumstances. Concentrations of adrenaline, vasopressin and ADP which together caused irreversible aggregation in heparinized PRP were insufficient to cause this response in citrated PRP from the same donor, in some cases, indicating a necessity for calcium in the potentiation mechanism. Any increase in aggregation in heparinized plasma was reduced by hirudin, indicating that thrombin can act as an additional agonist. It is suggested that the ability of adrenaline to potentiate platelet aggregation by physiological agents such as ADP and thrombin provides a mechanism for facilitating platelet activation at sites of vascular injury.

3.1. INTRODUCTION

The study of the effects of several different agonists on platelet aggregation is of interest because it reflects more closely the *in vivo* platelet environment in two aspects. Firstly, the circulating blood contains several agonists which can influence the platelet at any one time. Secondly, most of these agonists may not be raised above normal levels in the circulation in a given situation. Use of multiple agonists allows lower concentrations of each agonist to be tested for effects on platelet aggregation. In studies using single agonists the concentrations required are well in excess of physiological concentrations.

The phenomenon of positive interaction of low concentrations of agonists with platelets was first shown by O'Brien (1964). Ardlie *et al.*(1966) first described the effect of prior exposure to catecholamines, adrenaline, noradrenaline and dopamine. Catecholamines potentiated the response to ADP *in vitro* which suggested that the role of catecholamines in the initiation and subsequent development of thrombi deserved further investigation.

Aggregating agents were studied further in paired combination by Niewiarowski and Thomas (1966), Mills and Roberts (1967), Baumgartner and Born (1968), Nakanishi *et al.*(1971), Packham *et al.*(1973), Michal and Motamed (1976) and Grant and Scrutton (1980a). No multiple agonist studies have been undertaken.

The work of Huang and Detwiler (1981) contributed little to our understanding of the mechanisms of potentiation by particular agonists. Their work was based on varying the concentrations of different pairs of either weak or strong agonists. They concluded that the concentration of agonist determined the characteristics of the response regardless of the order of addition of the agonists. Their conclusion differed from Grant and Scrutton (1980a) and they claimed that this was due to the fact that the latter workers always added the higher concentration of agonist second. This was not, in fact, the case and where the concentration of the second agonist was lower than the first, the aggregation tracing still showed more of the characteristics of the second agonist.

Further evidence that the order of addition is significant comes from Owen and Le Breton (1980) who showed that calcium uptake differed, depending on the order of addition of adrenaline and ADP. When ADP was added first there was measurable ^{45}Ca uptake but not when adrenaline was added first. The concentrations of each agonist were kept constant and the final extent of aggregation was the same, regardless of order of addition.

Two agonists investigated in the present study may be increased in the circulation under conditions of stress. Stress may be an important risk factor in coronary artery disease. Baseline levels of adrenaline reported by Dimsdale and Moss (1980) were 0.64nM (range 0.48-1.2nM) which could rise 3-fold during public speaking.

Noradrenaline could rise 3-fold during vigorous exercise and increased 50% during public speaking from baseline levels of 3.4nM (range 2.2-4.3nM). Pathological stress could increase adrenaline levels to 10nM (Grant and Scrutton, 1980a).

Grant and Scrutton (1980a) reported that vasopressin increased 10-fold in adrenocortical insufficiency, from normal levels of 0.1nM but Johnston et al.(1981) quote normal levels of 1-5pM. The level of 5-hydroxytryptamine in blood is 0.1-10μM (De Clerck and Herman, 1983) which would be locally increased once platelet release has been activated. ADP circulating levels are unknown and it is also not known what a pathological level of ADP would be. The work on positive interaction of platelet agonists has, so far, only reported effects at concentrations higher than pathological levels. The lowest concentrations at which adrenaline and vasopressin interacted postively were at least two orders of magnitude greater than the blood concentrations of these hormones achieved in pathological states (Grant and Scrutton, 1980a). To be able to achieve potentiation of aggregation with at least pathological levels would be a major step in accepting in vitro aggregation as a proper reflection of in vivo phenomena.

The present work extends the study of the interaction of aggregating agents by challenging platelets with up to four agonists in immediate succession. It was suspected that an additional agonist, thrombin, would be present as a very low concentration of heparin was used as

anticoagulant. In these ways, it was anticipated that the study approximate more closely the situation in circulating blood. Heparan sulphate associated with vascular endothelium may be a physiologic regulator of blood coagulation (Rosenberg, 1977) so that heparin can be regarded as a more 'natural' anticoagulant than citrate (see section 2.1.1.2 for a comparison of the use of heparin versus citrate).

The study does not attempt to uncover a mechanism for the potentiation by any of the agonists. The aim of the study was to find out how low a concentration of an agonist can be used in vitro. In particular, a verification of the importance of circulating levels of catecholamines in platelet activation was sought. The lowest possible concentration of heparin was used to achieve anti-coagulation. This might allow thrombin generation. The platelets sometimes showed increasing sensitivity during the course of a single experiment but each result was controlled by observing, simultaneously, the response obtained in the absence of the final agonist.

3.2 METHODS AND MATERIALS

Blood was obtained and plasma prepared as described in Chapter 2. 1.3U/ml heparin was used as the anticoagulant for these experiments. The stress of venepuncture was minimized by allowing the donor to lie down and by the use of a winged infusion set (21Gx3/4") with a 12" (305mm) extension tube. The first two drops

were discarded and withdrawal of the blood with a syringe was continued without a tourniquet.

Initially heparin (CSL) (161.8U/mg) was used but was found to allow microaggregates to form after centrifugation with some donors. This type of heparin was reported (Han and Ardlie, 1974) as being pro-aggregatory at 8.3U/ml when added to washed platelets. Thereafter, liquid heparin from Allen and Hanbury's was used in this study with more satisfactory anticoagulation.

Since the stimulation by each agonist is not instantaneous and is only transitory, agonists were added at 15sec. intervals for an optimal rate of primary response for the range of concentrations used(Grant and Scrutton, 1980). The order of addition of agonists was kept constant,firstly adrenaline, then serotonin(5-hydroxytryptamine),noradrenaline or vasopressin and lastly ADP.

Thrombin in PRP was determined using the chromogenic substrate Chromozym TH. 770µl TRIS imidazole buffer (pH 8.4; 0.2M in 0.125M NaCl),10µl PRP (or thrombin) and 20µl Chromozym TH (187.5µM) were incubated in a cuvette at 37°C and the absorption per min (at 405nm) was recorded.

ADP, noradrenaline, adrenaline, 5-hydroxytryptamine (5HT), vasopressin, heparin and hirudin were obtained and stored as described in 2.2.

3.3 RESULTS

3.3.1 The Use of Lower Concentrations of Heparin to Prepare Platelet Rich Plasma.

As discussed in Chapter 2, the characteristics of platelet aggregation induced in heparinized plasma have been found to differ by different authors. Observations were therefore made with different concentrations of heparin and these were compared with citrate.

3.3.1.1 Effect of Heparin Concentration on Biphasic Aggregation due to ADP

As the heparin concentration for anticoagulation was increased, so the threshold concentration of ADP required to cause a biphasic response increased(Fig 3.1). This reduced sensitivity to ADP was further illustrated when the concentration of heparin was increased such that only a single phase occurred in response to 5μM ADP(Fig 3.2).

3.3.1.2 Comparison of Citrate and 1-2U/ml Heparin

Heparinized platelet rich plasma was not always more sensitive to ADP than was citrated plasma. The pattern of response varied from donor to donor. Further, not all donors exhibited a biphasic ADP response in either citrate or heparin. Of those that did, the threshold concentration for induction of biphasic aggregation in response to ADP was often the same for citrate and 1-2U/ml heparin. Of eleven donors tested, four exhibited greater sensitivity in heparin (Table 3.1). For each of these donors, the extent of primary aggregation in response to ADP was greater in PRP containing heparin (1-2 units/ml) than in PRP

anticoagulated with citrate. Furthermore, vasopressin (4μM) caused maximal aggregation in heparinized PRP, but almost no aggregation in citrated PRP. These differences could not be accounted for by differences in platelet count, since these were not significant.

Table 3.1 Comparison Between Threshold Concentrations of ADP for Induction of Biphasic Aggregation in Citrated and Heparinized (1-2U/ml) PRP.

Donor No	ADP threshold (μM)	
	citrated PRP	Heparinized PRP
1	2.7	1.8
2	1.8	1.8
3	5.4	5.4
4	7.2	7.2
5	8.1	7.2
6	1.8	1.8
7	3.6	1.8
8	5.4	5.4
9	4.5	4.5
10	4.5	4.5
11	4.5	2.7

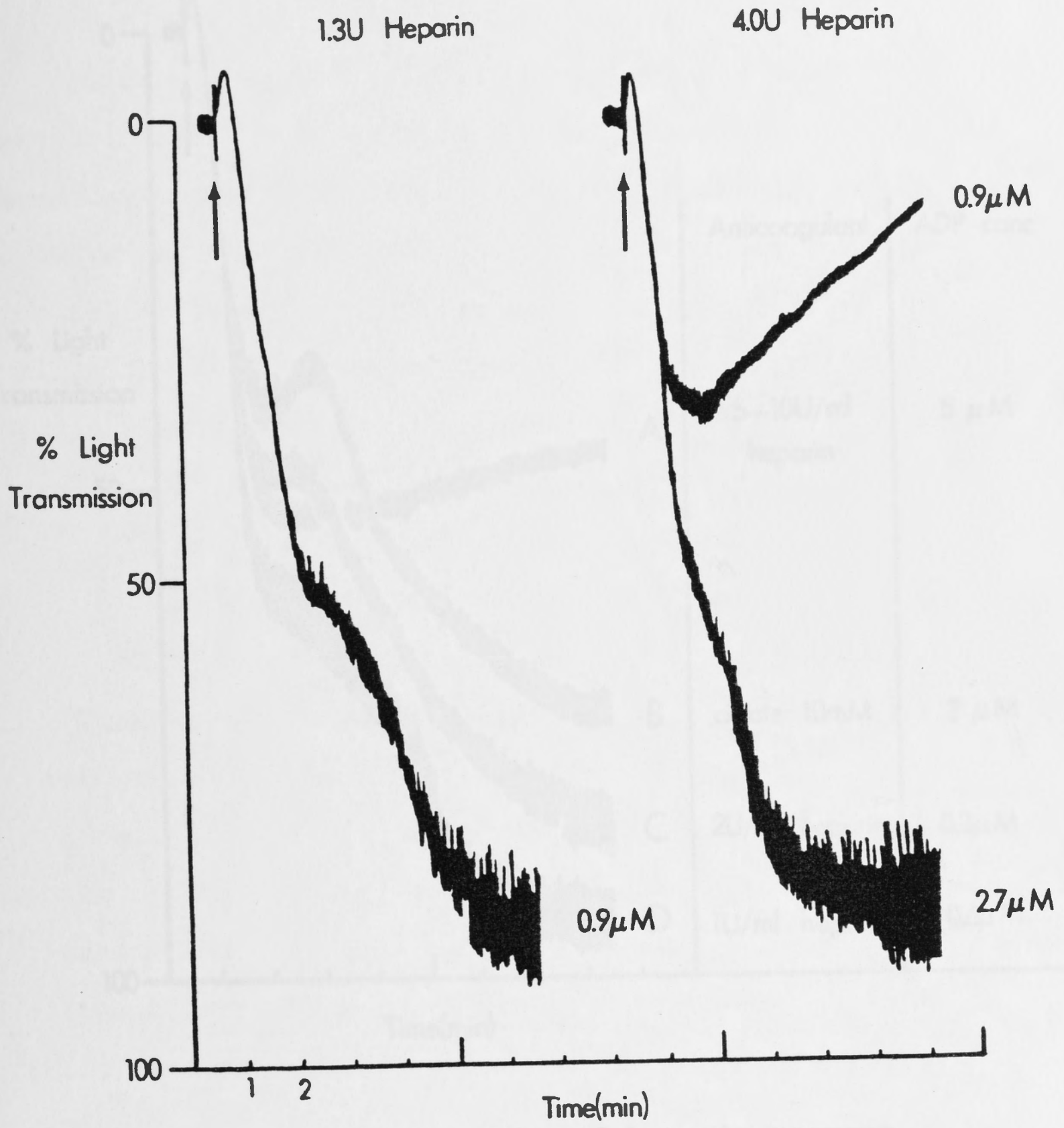

Figure 3.1 The effect of heparin concentration on ADP threshold.

The biphasic aggregation responses to adenosine diphosphate (ADP) are shown. Platelet rich plasma (PRP) was anticoagulated with 1.3U/ml and 4.0U/ml heparin, the latter concentration requiring more ADP to achieve the biphasic response.
The arrow indicates the time of addition of the ADP.

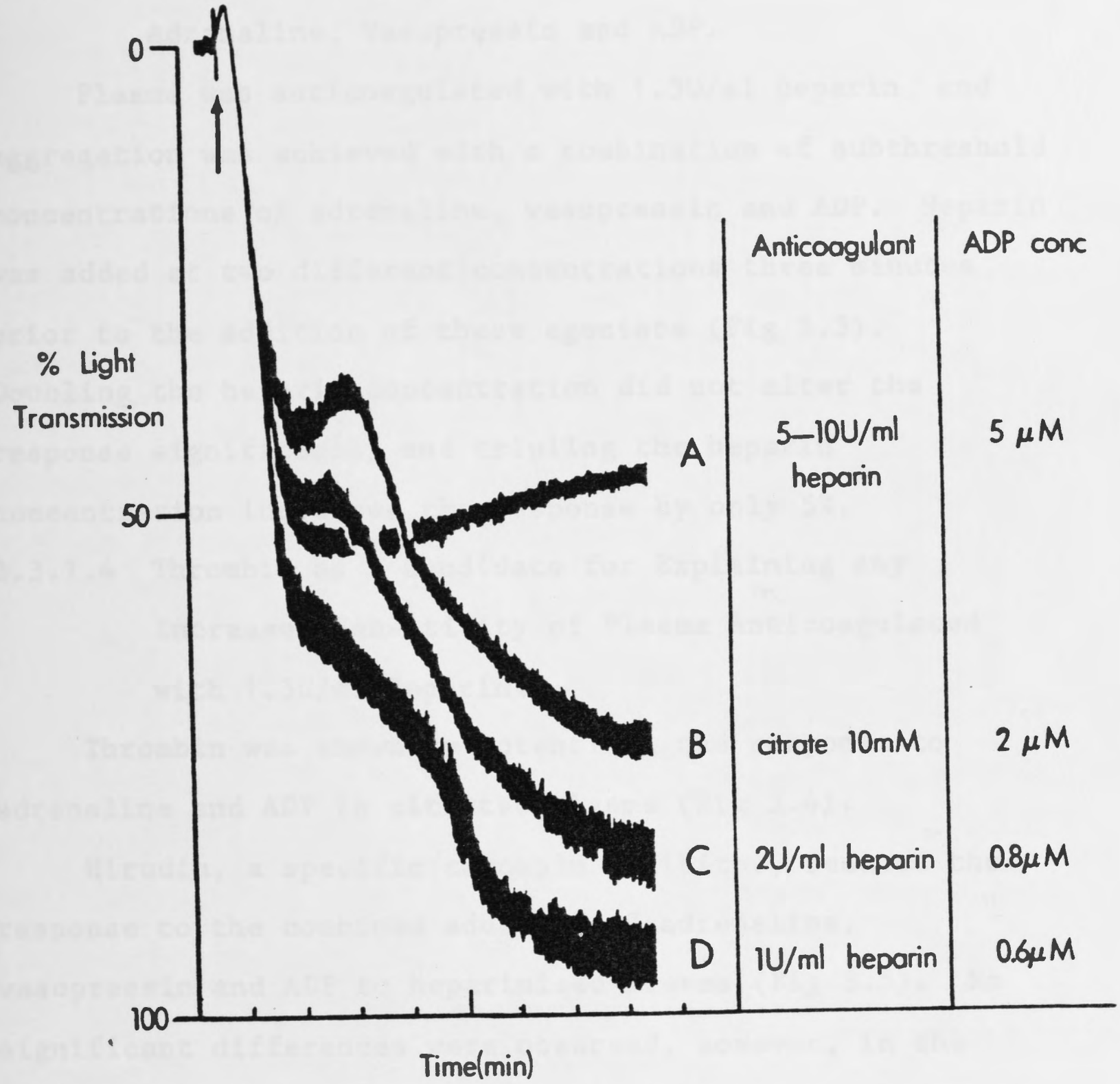

Figure 3.2 High concentrations of heparin prevent biphasic aggregation.

Platelet rich plasma from the same donor was anticoagulated with different concentrations of heparin (as shown) or citrate (10mM). ADP was added at increasing concentrations until biphasic aggregation was achieved. For concentrations of heparin of 5U/ml and higher, biphasic aggregation was inhibited for ADP (5μM).
The threshold heparin concentration for this inhibition varied between donors. Traces B, C and D were associated with release of ATP. The arrow indicates the time of addition of ADP. Platelet counts varied from 290,000-330,000/μl.

3.3.1.3 Effect of Added Heparin on the Interaction between Adrenaline, Vasopressin and ADP.

Plasma was anticoagulated with 1.3U/ml heparin, and aggregation was achieved with a combination of subthreshold concentrations of adrenaline, vasopressin and ADP. Heparin was added at two different concentrations three minutes prior to the addition of these agonists (Fig 3.3). Doubling the heparin concentration did not alter the response significantly and tripling the heparin concentration increased the response by only 5%.

3.3.1.4 Thrombin as a Candidate for Explaining any Increased Sensitivity of Plasma Anticoagulated with 1.3U/ml Heparin.

Thrombin was shown to potentiate the response to adrenaline and ADP in citrated plasma (Fig 3.4).

Hirudin, a specific thrombin inhibitor, reduced the response to the combined addition of adrenaline, vasopressin and ADP to heparinized plasma (Fig 3.5). No significant differences were observed, however, in the thrombin levels (0-0.06U) using the chromogenic substrate, Chromozym TH in citrated or heparinized PRP from the same donor.

3.3.1.5 Discussion on the Use of Heparin.

Higher concentrations of heparin tend to increase the threshold for ADP which could be explained by inhibition of any thrombin which may be present. The low concentration of 1.3U/ml heparin sometimes increased sensitivity when compared to responses in citrate, possibly due to a pro-aggregatory effect. It is probable that citrate inhibits vasopressin by reducing the concentration of calcium ions

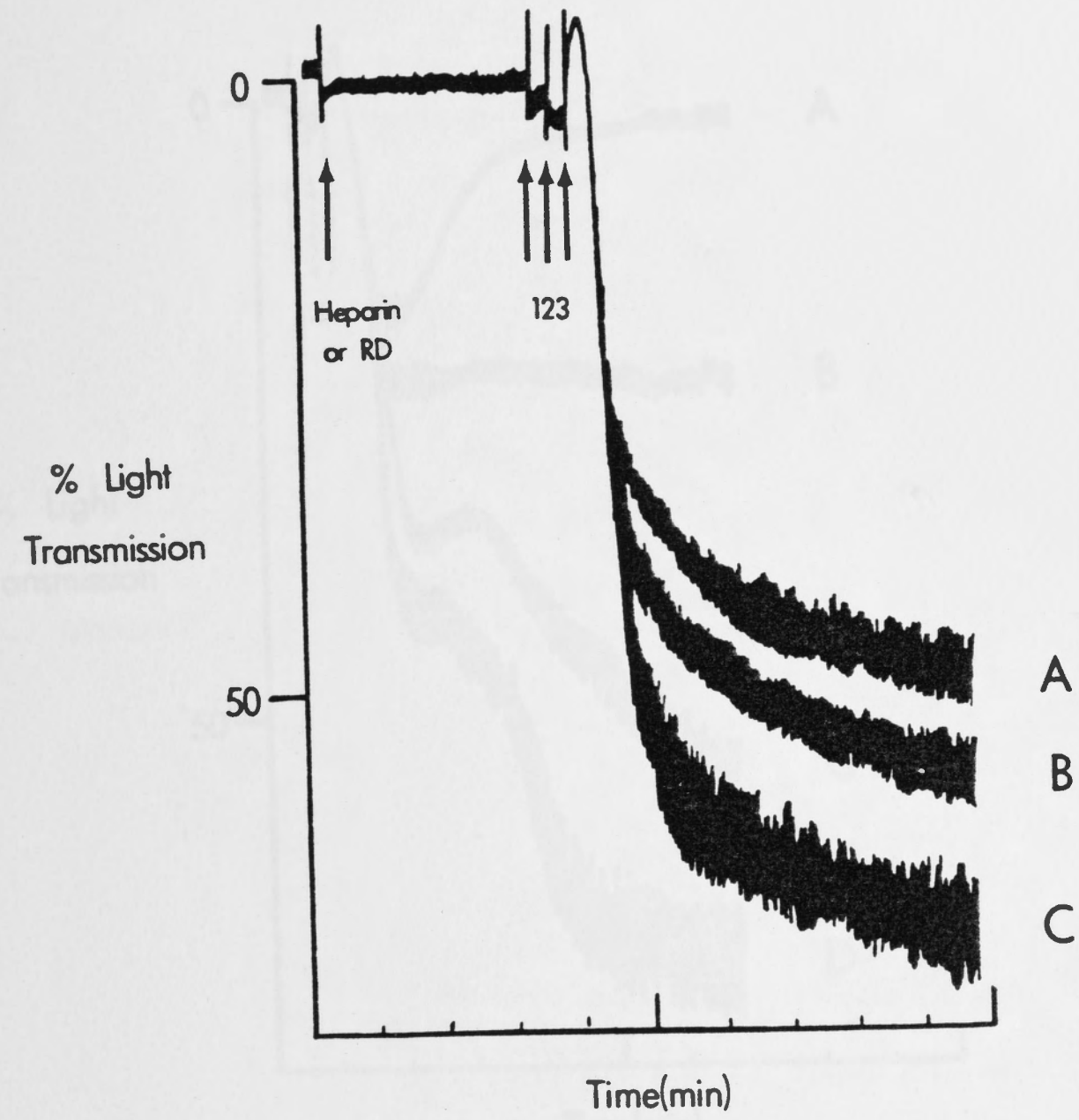

Figure 3.3 The effect of added heparin on the response to the combined addition of adrenaline, vasopressin and ADP.

The platelet count was 295,000/µl in PRP anticoagulated with 1.3U/ml heparin. In another experiment 3.5U/ml extra heparin did not alter the response at all. The arrows indicate the time of addition of :

1. adrenaline (22nM)
2. vasopressin (2nM) and
3. ADP (435nM).

A. no added heparin (RD).
B. 1.7U/ml extra heparin.
C. 3.5U/ml extra heparin.

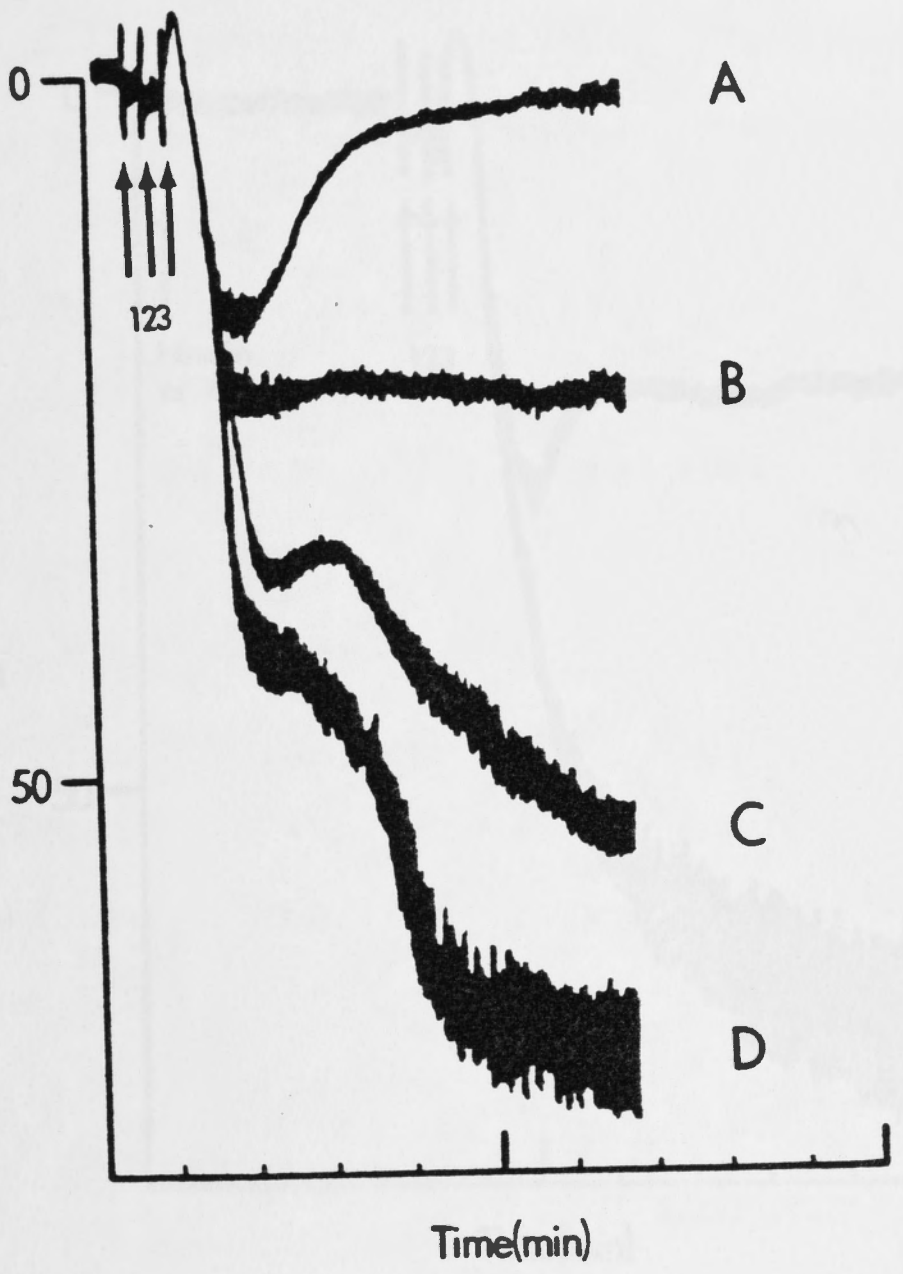

Figure 3.4 The potentiation of adrenaline and ADP by thrombin in PRP anticoagulated with 10mM citrate.

A. RD + 4nM adrenaline + 0.35μM ADP
B. RD + 2.6mU/ml thrombin
C. 2.6mU/ml thrombin + RD + 0.35μM ADP
D. 2.6mU/ml thrombin + 4nM adenaline + 0.35μM ADP
The arrows indicate the time of addition in the order:
1. thrombin or RD
2. adrenaline or RD
3. ADP.

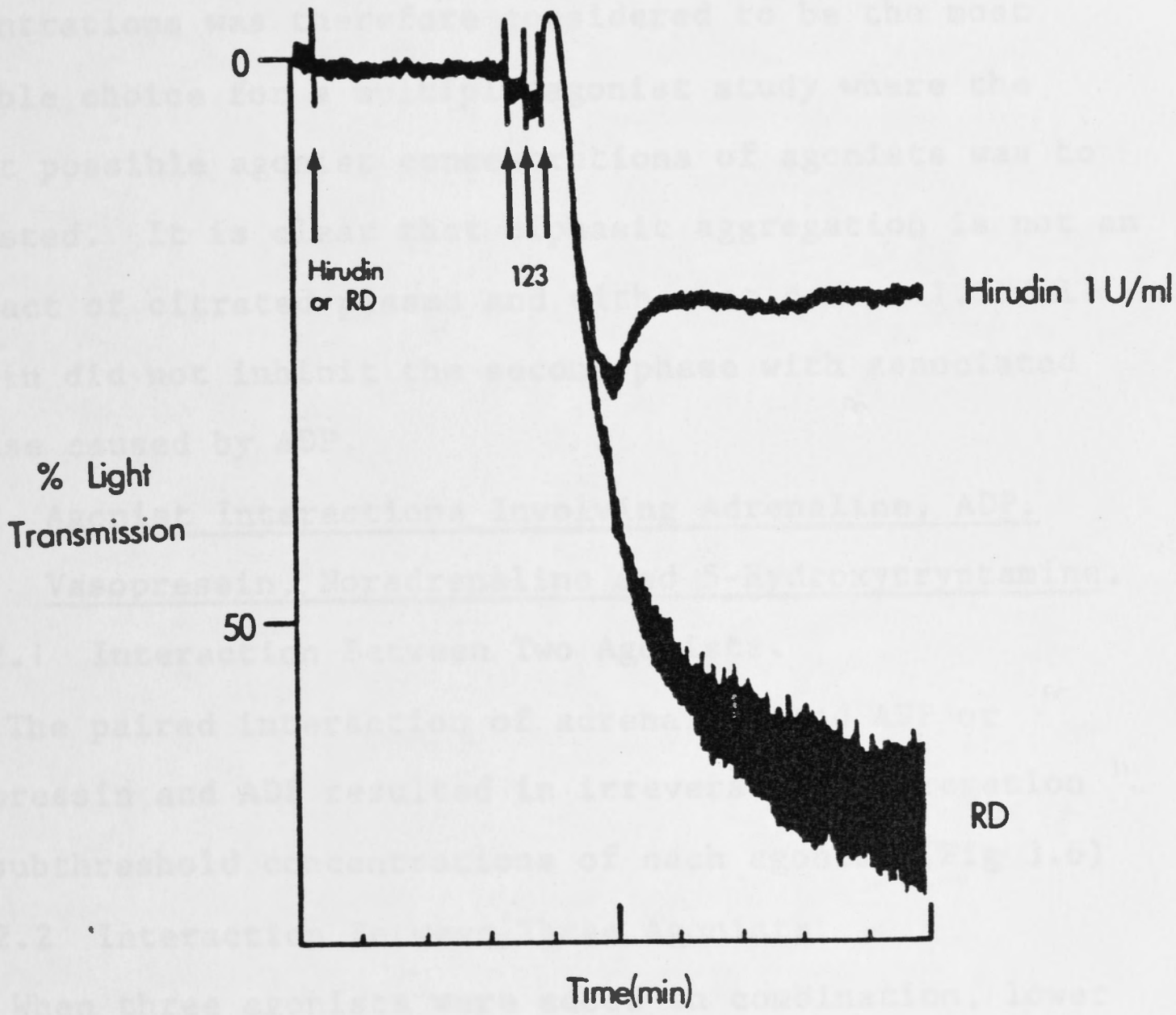

Figure 3.5 The effect of pre-incubation with hirudin (1U/ml), a specific thrombin inhibitor, on the response to adrenaline, vasopressin and ADP.

PRP was anticoagulated with 1.3U/ml heparin. The arrows indicate the time of addition of:

1. adrenaline 18nM
2. vasopressin 0.8nM
3. ADP 0.18μM.

(Haslam and Rosson,1972). For most donors, 1.3U/ml heparin did not increase the sensitivity to ADP at all. When three agonists were used, added heparin at 3 times the anticoagulating concentration caused a small increase in response in one experiment. Heparin at low (1-1.3U/ml) concentrations was therefore considered to be the most suitable choice for a multiple agonist study where the lowest possible agonist concentrations of agonists was to be tested. It is clear that biphasic aggregation is not an artifact of citrated plasma and with most donors 1.3U/ml heparin did not inhibit the second phase with associated release caused by ADP.

3.3.2 Agonist Interactions Involving Adrenaline, ADP, Vasopressin, Noradrenaline and 5-Hydroxytryptamine.

3.3.2.1 Interaction Between Two Agonists.

The paired interaction of adrenaline and ADP or vasopressin and ADP resulted in irreversible aggregation for subthreshold concentrations of each agonist (Fig 3.6)

3.3.2.2 Interaction Between Three Agonists

When three agonists were added in combination, lower concentrations of each agonist were required to achieve irreversible aggregation. Fig 3.7 illustrates an example of the responses when 1U/ml heparin (CSL) was used, and Fig 3.8 illustrates an example of the responses when 1.3U/ml heparin (Allen and Hanburys) was used.

3.3.2.3 Interaction Between Four Agonists.

Noradrenaline and 5HT are also present in the circulation in addition to vasopressin, adrenaline and ADP.

With the addition of noradrenaline to the three agonists already used, even lower concentrations could achieve irreversible aggregation (Fig 3.9). The result was variable, however, and as much as 43nM noradrenaline was required for irreversible aggregation when using adrenaline (4.3nM), vasopressin (0.5 - 1nM) and ADP (0.2 - 0.4μM) in some donors.

5HT added in combination with adrenaline, vasopressin and ADP also showed variable responses. For some donors aggregation was always reversible, no matter how much 5HT added. The lowest concentration of 5HT capable of causing irreversible aggregation in combination with the other three agonists was 120nM (Fig 3.10). The lowest concentrations of each agonist which interacted positively when combined with other agonists are shown, together with their physiological and pathological levels, where known, in Table 3.2.

Table 3.2 Comparison of lowest concentration of agonist causing *in vitro* aggregation with reported physiological and pathological levels in circulating blood.

Agonist	Physiological Level	Pathological Level	Level to cause *in vitro* aggregation in this study
Adrenaline	1nM* (0.48-1.2nM)@	10nM*	2.6nM
Noradren.	2.2-4.3nM@		4.3nM
Vasopressin	1-5pM#	1nM*	0.5nM
5-HT	0.1-10μM⁰		120nM

* Grant and Scrutton (1980). @ Dimsdale and Moss(1980). # Johnston *et al*. (1981). ⁰ De Clerck and Herman(1983) The levels given causing *in vitro* aggregation are those achieved by the addition of agonists. No assay was done on the existing levels in the blood. The stress of venepuncture was minimized as described.

3.3.2.4 Effect of Citrate on the Response to Multiple Agonists.

The response to multiple agonists was decreased in PRP obtained from blood anticoagulated with citrate compared with PRP obtained from blood anticoagulated with heparin in several donors. The concentration of adrenaline needed to enhance the aggregation response in citrated plasma was at least two orders of magnitude greater than the minimum concentration which achieved amplification in plasma containing a low concentration of heparin. Less adrenaline was required as the concentration of ADP was increased.

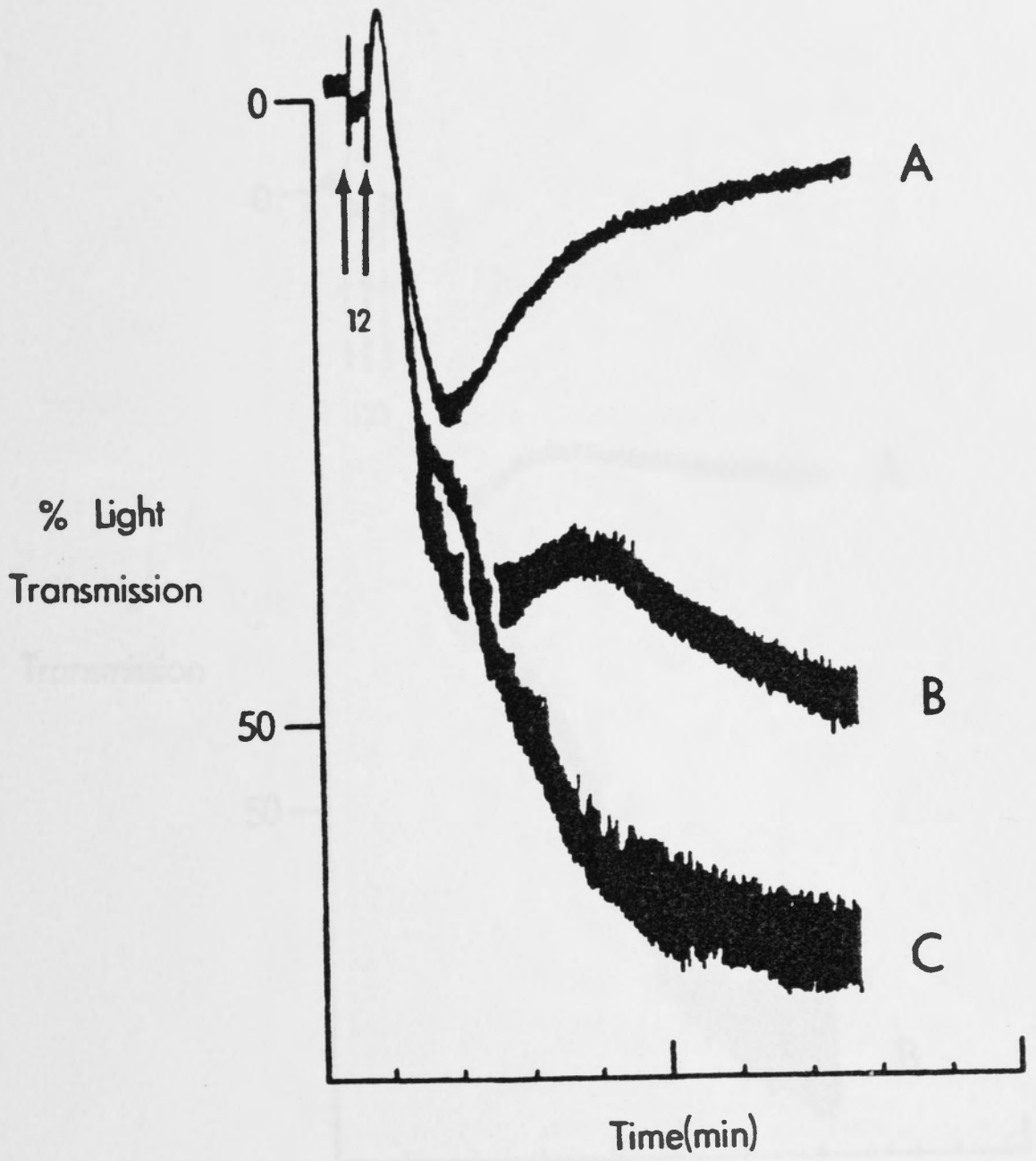

Figure 3.6 The positive interaction of adrenaline and ADP, vasopressin and ADP.

The PRP was anticoagulated with 1.2U/ml heparin. Neither 18nM vasopressin nor 42nM adrenaline caused aggregation when added by themselves. These responses were obtained 120 min after blood collection time. Platelet count was 300,000/µl. Agonists were added at the times indicated by the arrows which are 15sec. apart. The order of addition was as indicated below.
RD = reagent diluent

	Agonists 1.	2.
A.	RD	800nM ADP
B.	18nM vasopressin	800nM ADP
C.	42nM adrenaline	800nM ADP

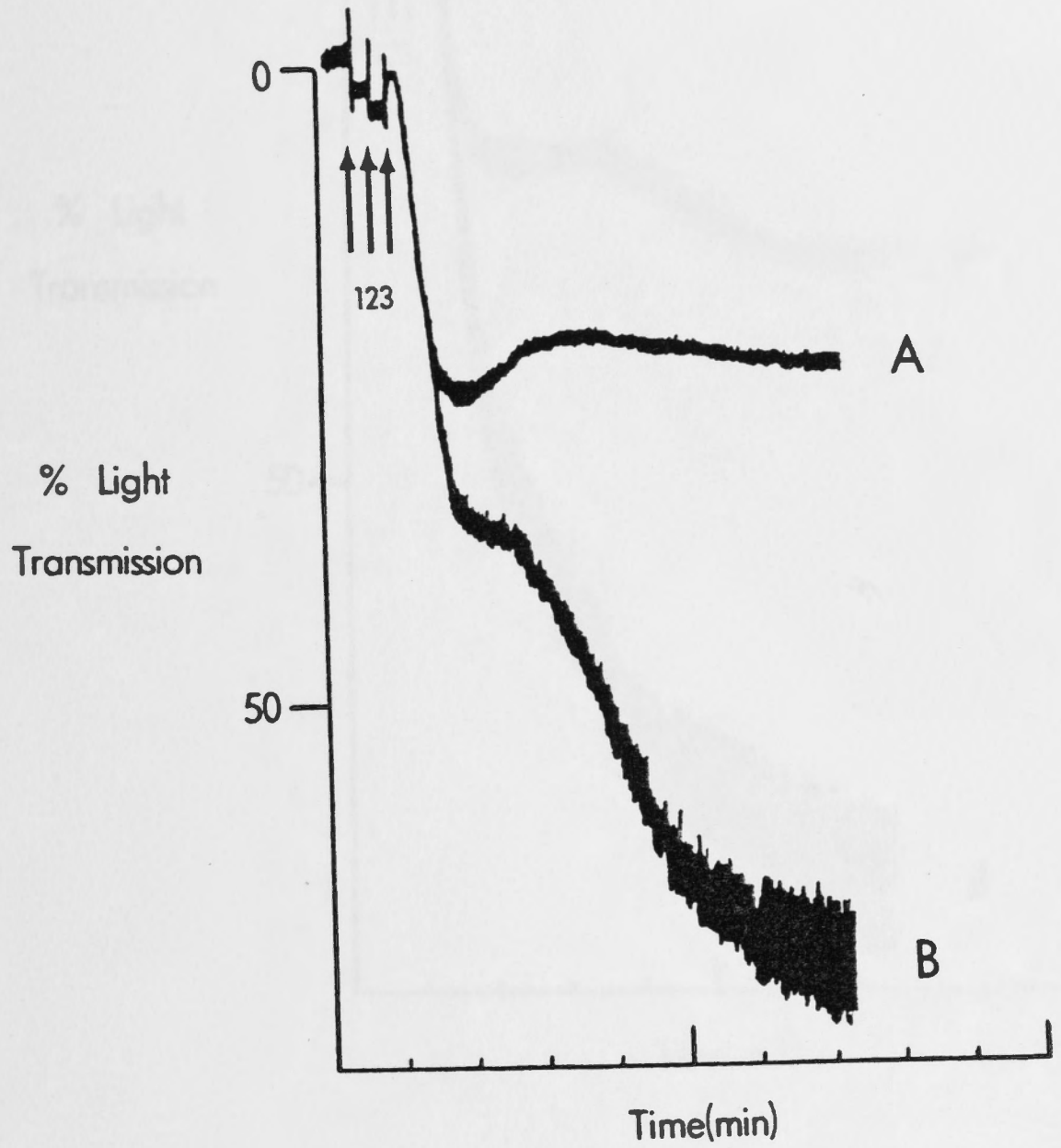

Figure 3.7 The positive interaction of adrenaline, vasopressin and ADP.

The PRP was anticoagulated with 1U/ml heparin (CSL). 2.5nM adrenaline did not cause aggregation by itself. These responses were obtained 200 mins after blood collection. Platelet count was 277,000/µl. Agonists were added at the times indicated by the arrows which are 15sec. apart. The order of addition was as indicated below.
RD = reagent diluent

	Agonists		
	1.	2.	3.
A.	RD	0.2nM vasopressin	200nM ADP
B.	3.5nM adrenaline	0.2nM vasopressin	200nM ADP

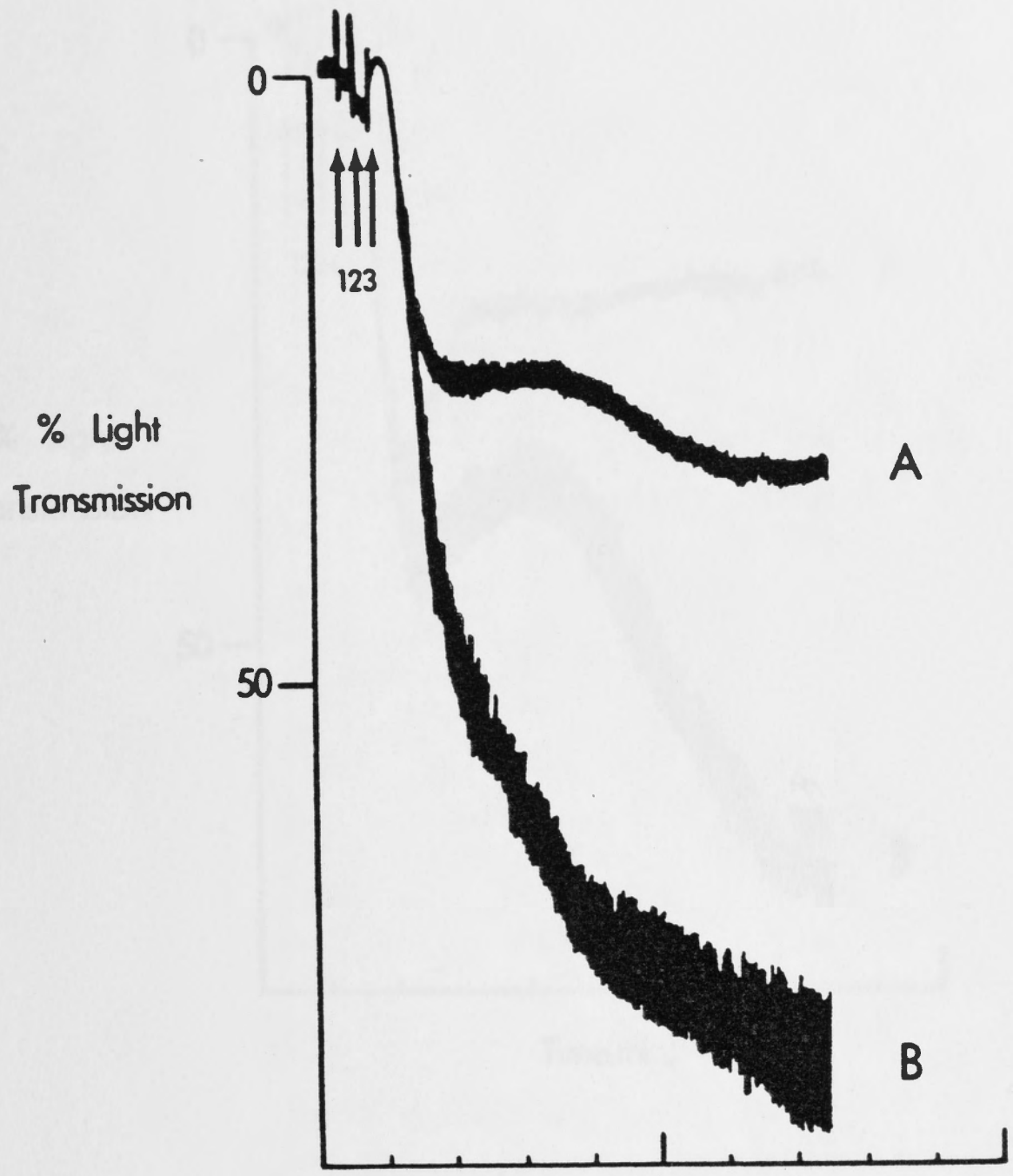

Figure 3.8 The positive interaction of adrenaline, vasopressin and ADP.

The PRP was anticoagulated with 1.3U/ml heparin (A + H). 21nM adrenaline did not cause aggregation by itself. These responses were obtained 70 mins after blood collection. Platelet count was 307,000/µl. Agonists were added at the times indicated by the arrows which are 15sec. apart. The order of addition was as indicated below.
RD = reagent diluent

	Agonists		
	1.	2.	3.
A.	RD	1.0nM vasopressin	200nM ADP
B.	21nM adrenaline	1.0nM vasopressin	200nM ADP

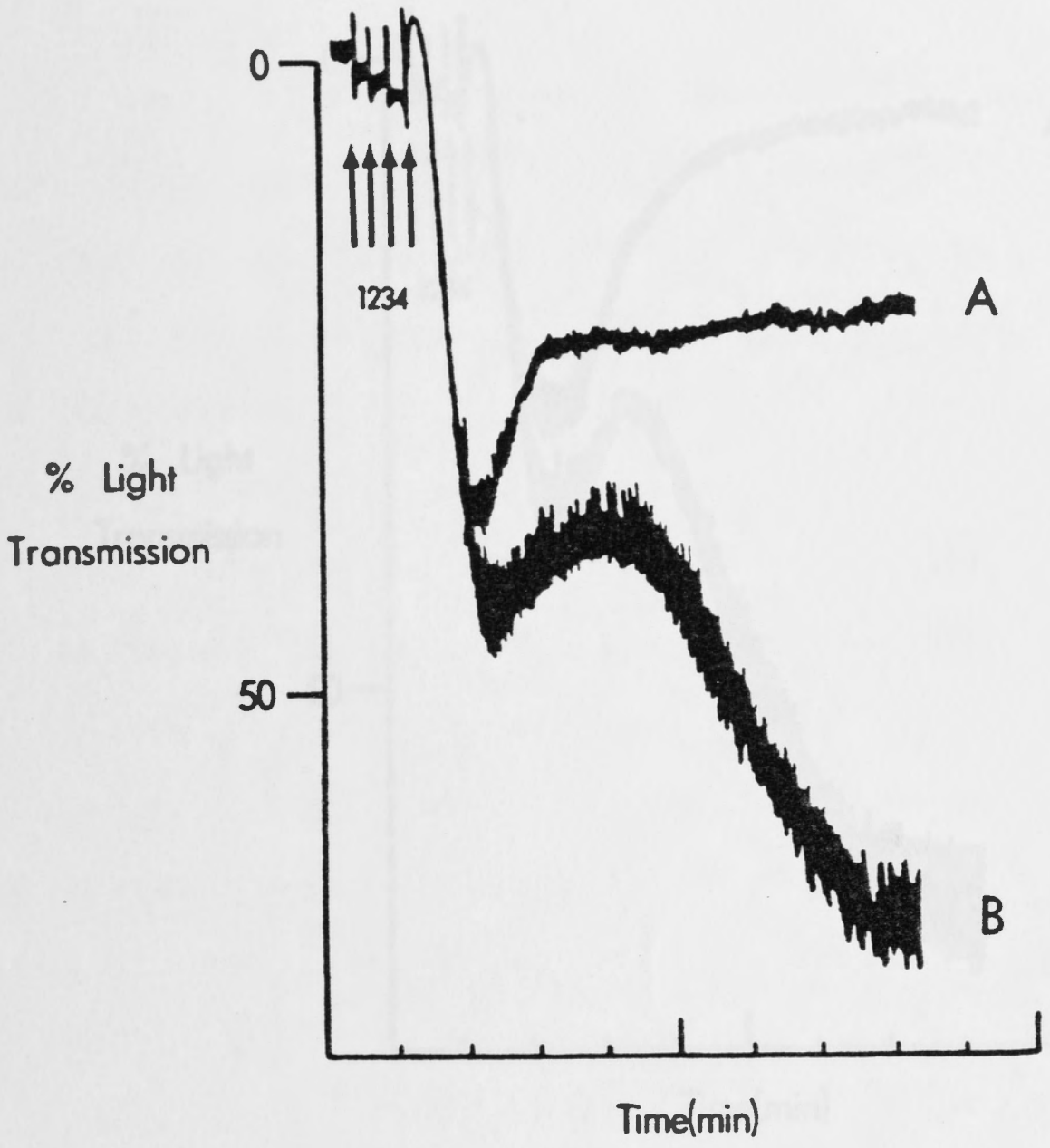

Figure 3.9 The positive interaction of adrenaline, noradrenaline, vasopressin and ADP.

The PRP was anticoagulated with 1U/ml heparin (CSL). 2.6nM adrenaline did not cause aggregation by itself. These responses were obtained 130 mins after blood collection. Platelet count was 319,000/µl. Agonists were added at the times indicated by the arrows which are 15sec. apart. The order of addition was as indicated below.
RD = reagent diluent

	Agonists			
	1.	2.	3.	4.
A.	RD	4.3nM noradrenaline	0.5nM vasopressin	400nM ADP
B.	2.6nM adrenaline	4.3nM noradrenaline	0.5nM vasopressin	400nM ADP

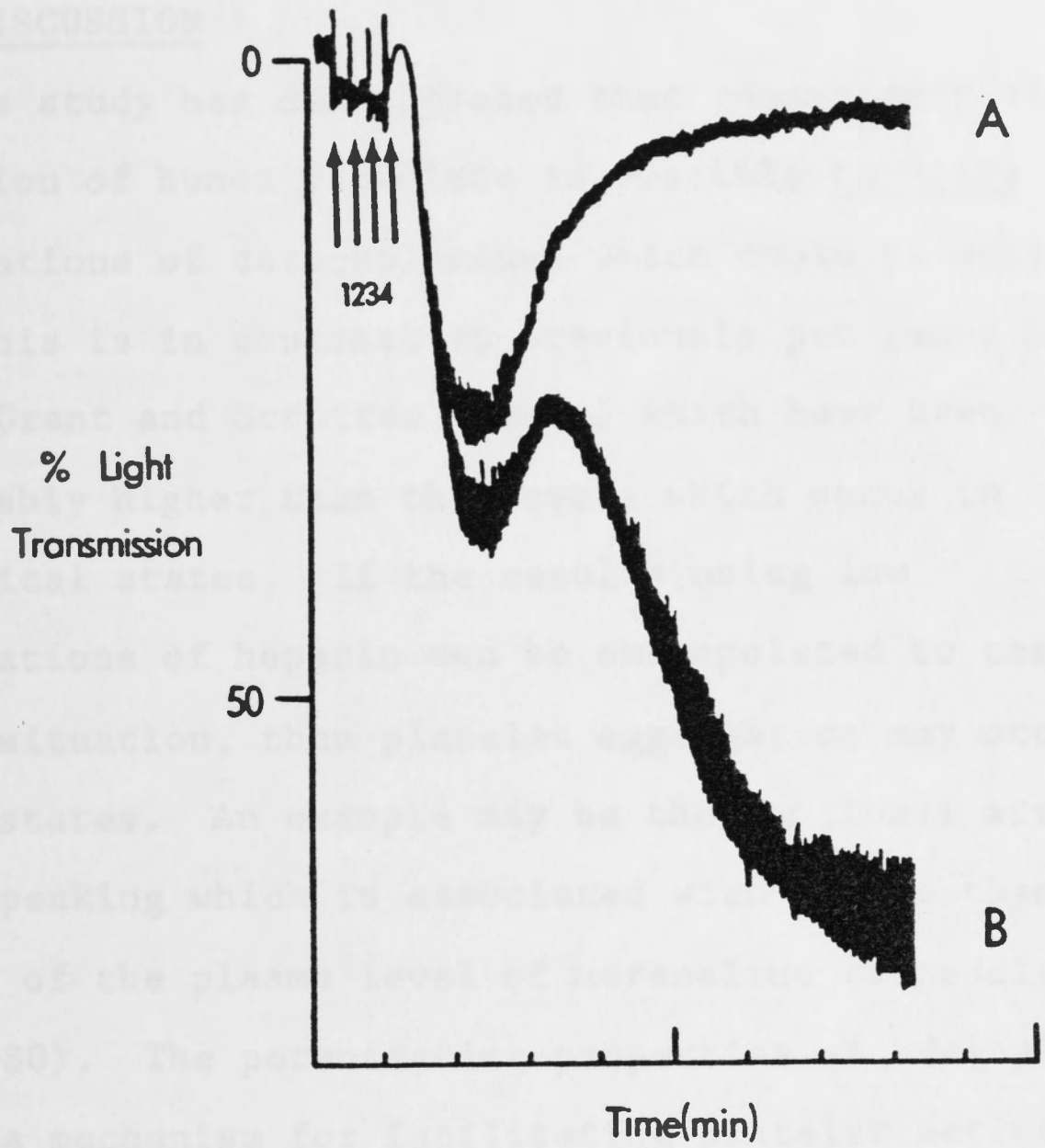

Figure 3.10 The positive interaction of adrenaline, vasopressin, 5-hydroxytryptamine and ADP.

The PRP was anticoagulated with 1.3U/ml heparin (A + H). 4.3nM adrenaline did not cause aggregation by itself. These responses were abtained 140 mins after blood collection. Platelet count was 291,000/µl. Agonists were added at the times indicated by the arrows which are 15sec. apart. The order of addition was as indicated in the right hand columns.
RD = reagent diluent

	Agonists			
	1.	2.	3.	4.
A.	RD	1.0nM vasopressin	120nM 5HT	200nM ADP
B.	4.3nM adrenaline	1.0nM vasopressin	120nM 5HT	200nM ADP

3.4.3 DISCUSSION

This study has demonstrated that enhancement of aggregation of human platelets is possible *in vitro* using concentrations of catecholamines which could be achieved *in vivo*. This is in contrast to previously published minimum levels (Grant and Scrutton, 1980a) which have been considerably higher than the levels which occur in pathological states. If the results using low concentrations of heparin can be extrapolated to the *in vivo* situation, then platelet aggregation may occur in certain states. An example may be the emotional stress of public speaking which is associated with a more than doubling of the plasma level of adrenaline (Dimsdale and Moss, 1980). The potentiating properties of adrenaline may provide a mechanism for facilitating platelet activation at sites of vascular injury.

The method used here for examining agonist interaction differs from others in that a much lower concentration of heparin was used to anticoagulate blood. With this method of collection, platelets were at least as sensitive as those collected in citrate, and often more sensitive to aggregating agents. Those platelets which were found to be more sensitive responded to multiple agonists at concentrations obtained in physiological and pathological states.

The reduction in the sensitivity of the heparinized PRP caused by hirudin, a specific inhibitor for thrombin, suggests that thrombin participates in the aggregatory

response. This has been suggested by earlier workers, Ardlie and Han (1974)and Huzoor-Akbar and Ardlie (1976; 1977) who particularly emphasized a role for thrombin in the release reaction. The lack of difference in thrombin levels in PRP anticoagulated with either citrate or heparin seems to negate this theory. Nevertheless, a small amount of thrombin may act as an additional agonist, inhibitable by hirudin. MacIntyre et al.(1981) reported that heparin potentiates aggregation, but in the present study heparin had to be increased 3-fold above the anticoagulant concentrations used to cause any enhancement of aggregation caused by three combined agonists.

The reduced effectiveness of adrenaline in the presence of citrate is consistent with calcium fluxes being involved in the mechanism of adrenaline potentiation (Owen et al., 1980). Others have obtained evidence, however, to show that adrenaline does not cause net influx of calcium into human platelets unless secretion occurs(Brass and Shattil,1982; Clare and Scrutton,1984). An alternative explanation for the effect of citrate may have to be sought, not necessarily, however, excluding effects on membrane bound calcium. In addition, and consistent with observations made by Haslam and Rosson (1972), citrate appears to inhibit vasopressin which therefore, probably requires calcium for its action.

There are possible similarities between the test system described for this study and physiological

anticoagulation. Anti-thrombin is considered to be an important natural anticoagulant and its interaction with clotting factors is significantly enhanced by heparin (Rosenberg, 1977). Heparan sulphate, which is associated with endothelial cells, may be a normal cofactor for antithrombin in regulating coagulation. In both the test system described in the present study and in the haemostatic response (Shattil and Bennett, 1980) platelet activation is enhanced by thrombin. Furthermore, the interaction of platelets with a combination of agonists probably more closely reflects platelet activation <u>in vivo</u>.

CHAPTER 4

MECHANISM OF THE FACILITATING EFFECTS OF ADRENALINE ON PLATELET AGGREGATION

4.0 SUMMARY

Adrenaline, by itself, can aggregate human platelets *in vitro*, and also has the property of being able to potentiate the aggregation response to subthreshold concentr ations of other platelet agonists. Since it is largely inhibited by aspirin, the ability of adrenaline, alone, to cause platelet aggregation in citrated plasma is believed to depend on the cyclooxygenase pathway of arachidonic acid metabolism. This chapter examines whether the potentiating effects of adrenaline also depend on this mechanism.

This report describes the effects of several inhibitors of arachidonate metabolism on adrenaline-potentiated aggregation and TXB_2 production. It was shown that adrenaline stimulated a cyclooxygenase-independent pathway resulting in potentiation of platelet aggregation. Adrenaline also stimulated the cyclooxygenase pathway to an extent sufficient to generate TXB_2 when platelets were under the inhibitory influence of aspirin, but only upon the addition of large concentrations of arachidonate. Adrenaline was not able to cause this stimulation in the presence of indomethacin and BW755C, a dual cyclooxygenase and lipoxygenase inhibitor. At the concentrations of these inhibitors used, adrenaline was unable to potentiate arachidonate-induced aggregation, but was able to potentiate ADP-induced aggregation. There appears to be at least two mechanisms by which adrenaline can potentiate platelet aggregation, one of these being cyclooxygenase-

independent,the relative importance of which may depend on the function of this pathway in the aggregation response of the principal agonist. In the absence of thromboxanes, more adrenaline is required to potentiate aggregation.

The facilitating effect of adrenaline on a cyclooxygenase-independent pathway was mimicked by clonidine, a partial α_2-receptor agonist but not by phenylephrine an α_1-agonist. This indicates that these effects of adrenaline on the platelet occur via stimulation of the α_2-receptor. Dibenamine and phenoxybenzamine, post-receptor inhibitors, inhibited even high concentrations of adrenaline and inhibited the second phase of the ADP-response. Not only did these inhibitors prevent adrenaline potentiation of ADP, but the response of the combined agonists in their presence, was less than the ADP response alone. The biochemical changes subsequent to receptor occupation are investigated further in later chapters.

4.1 INTRODUCTION

Potentiation of human platelet aggregation by adrenaline may possibly represent a prime role for this catecholamine in the haemostatic response to injury of a blood vessel. Increased levels of this catecholamine may enable platelets to aggregate more quickly and irreversibly to plug a wound. It is not known whether adrenaline, by itself, can cause aggregation *in vivo* and it is disputed whether the observed *in vitro* aggregation in citrated PRP is an artifact or not. Adrenaline has been

reported, however, by several workers as being able to potentiate the platelet aggregation response to other agonists in both citrated and heparinized plasma (O'Brien, 1964; Ardlie et al., 1966; Mills and Roberts, 1967; Grant and Scrutton, 1980a).

The ability of adrenaline alone to cause platelet aggregation in vitro, is reported by Best (1980) to be largely dependent on the metabolism of arachidonic acid via the cyclooxygenase pathway to thromboxane A_2(TXA_2), the first phase, which is independent of thromboxane, being very small. Both aspirin and n-butyl imidazole, which inhibit cyclooxygenase and thromboxane synthetase respectively, prevent the second phase of adrenaline-induced aggregation. It is of interest to determine whether the ability of adrenaline to potentiate aggregation by other agonists also depends on the metabolism of arachidonic acid.

Rao et al. (1980a) using a thin layer chromatography method, have previously shown that adrenaline can overcome aspirin inhibition of ADP-, and arachidonate-induced aggregation without utilizing the cyclooxygenase pathway, as shown by lack of TXA_2 production. This is not unexpected since aspirin is believed to irreversibly acetylate the enzyme cyclooxygenase, but the mechanism by which adrenaline achieves potentiation independently of prostaglandin synthesis remains largely unexplored.

This study sought to verify first of all that the mechanism of adrenaline potentiation could, in part, be

thomboxane independent. The effects of different cyclooxygenase inhibitors on adrenaline potentiation of ADP- and arachidonate-induced aggregation was examined. The effectiveness of these various inhibitory agents on thromboxane synthesis was determined using a radioimmunoassay technique. Clonidine, a partial α_2-receptor agonist, and phenylephrine (a partial α_1-agonist) were also studied to determine whether they were able to potentiate aggregation in a similar manner to adrenaline in the absence or presence of these inhibitors.

Following the establishment of the involvement of a thromboxane-independent pathway in adrenaline potentiation, certain aspects of the α-receptor system itself were considered to be worthy of further study. Dibenamine and phenoxybenzamine are thought to be drugs which act on the α-receptor system but by a mechanism independent, to some extent, of agonist binding (Lullman et al., 1969; Mitchelson, 1975). Their actions have been found to be different, despite similarities in structure between the two drugs (Jafferji and Michell, 1976; Cockcroft et al.,1980). Dibenamine seems to inhibit calcium mobilization without altering phosphatidylinositol turnover which is, however, affected by phenoxybenzamine. It was thought, therefore, that the effects of these two drugs on the adrenaline-potentiated response in platelets might reveal more of the events following α-receptor activation by adrenaline. The results indicate that further attention

needs to be focussed on platelet membrane events subsequent to adrenaline α-receptor interaction.

4.2. METHODS AND MATERIALS

Platelet aggregation, release of ATP and TXB_2 formation were assessed utilizing techniques described in Chapter 2. Blood was anticoagulated with citrate.

The agonists used were adrenaline (Calbiochem), adenosine diphosphate (ADP)(Sigma), arachidonic acid (Sigma), clonidine (Boehringer-Ingelheim) and phenylephrine (Sigma).

The inhibitors used were acetylsalicylic acid (ASA)(aspirin) (BDH), indomethacin (Sigma),3-amino-1-[m-(tri-fluoromethyl)-phenyl]-2-pyrazoline (BW755C)(Wellcome), n-butyl-imidazole (Koch-Light), phenoxybenzamine and dibenamine (Smith, Kline and French).

Aspirin,100μM, unless otherwise stated was preincubated with PRP in a tightly stoppered syringe at 37^{0}C for at least 10min prior to the test. The other inhibitors were added to the PRP in the cuvette 1min prior to the addition of agonists.

4.3. RESULTS

4.3.1 Potentiation of ADP-induced Aggregation by Adrenaline

The synergistic effect on agregation of subthreshold concentrations of ADP and adrenaline was associated with a small increase in TXB_2 (Fig 4.1).

The appearance of the potentiated aggregation response varied from donor to donor. The tracing took on the appearance of an adrenaline-induced aggregation response in some cases. That is, the "first phase" was small and there was a lag before a second phase. Nevertheless, there was also a shape change. This tended to occur when low concentrations of both ADP and adrenaline were used, with the order of addition being adrenaline before ADP.

Pre-incubation with 100μM aspirin did not prevent adrenaline potentiation of ADP-induced aggregation but did prevent TXB_2 formation (Fig 4.2). In the presence of aspirin, higher concentrations of adrenaline were required to acheive a similar degree of potentiation observed without aspirin. For example, the addition of 0.8μM adrenaline and 1.6μM ADP in the presence of aspirin (Fig4.2,tracingD) resulted in the same extent of aggregation as 0.08μM adrenaline and 1.6μM ADP in the absence of aspirin in the same donor(not shown). Nonetheless, in the presence of aspirin, 0.08μM adrenaline clearly potentiated 1.6μM ADP(Fig.4.2,tracingC).

When PRP was pre-incubated with 10-100μM indomethacin for 1 min, potentiation of the first phase of the ADP response was also possible(Fig 4.3.) In the presence of 100μM indomethacin, the addition of 8μM adrenaline was necessary to obtain marked potentiation of aggregation but without TXB_2 formation. Similarly, when the dual cyclooxygenase and lipoxygenase inhibitor, BW755C (38μM) was used, enhancement of ADP-induced aggregation by

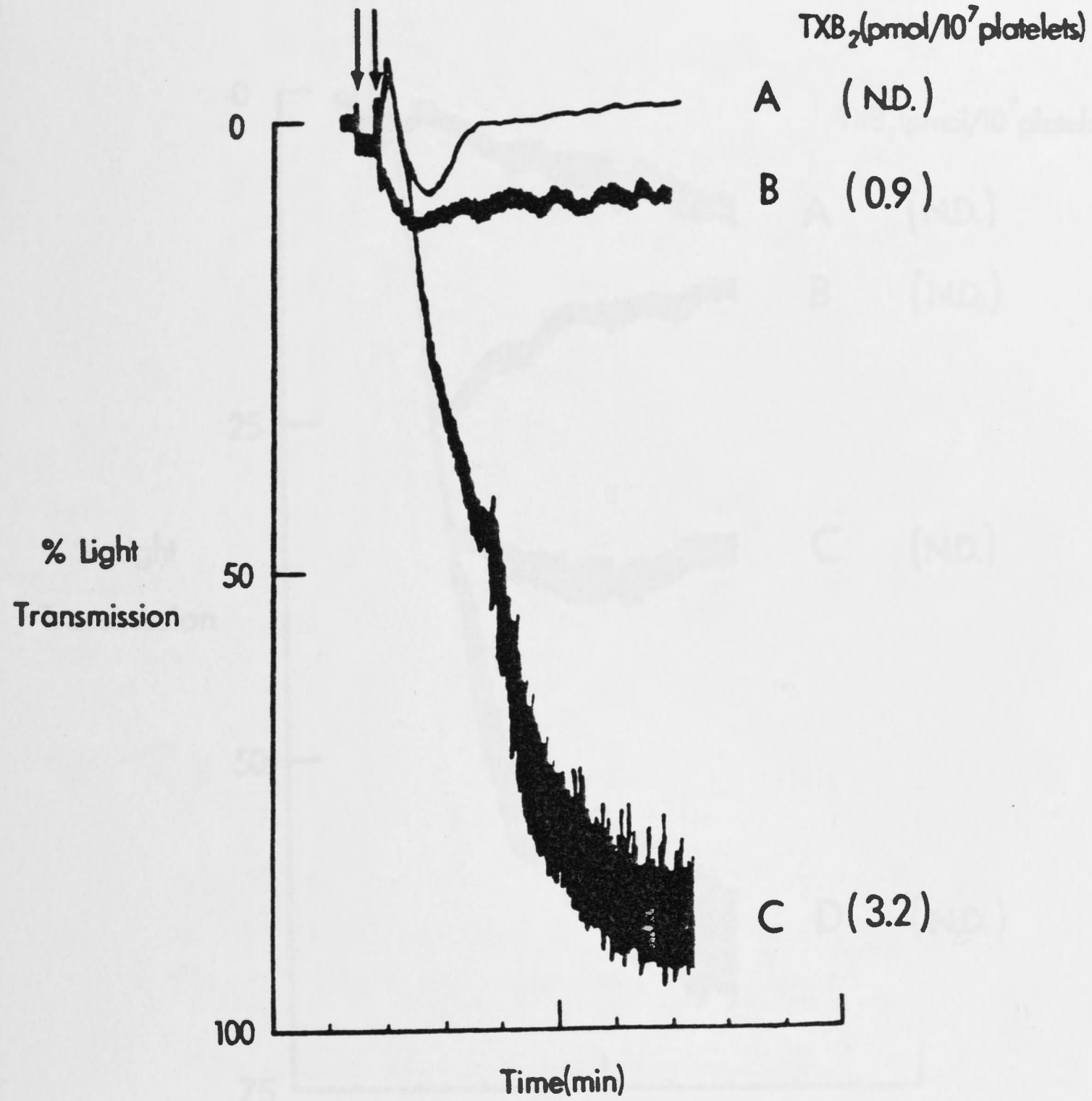

Figure 4.1 Potentiation of ADP-induced aggregation by adrenaline.

A. RD + 0.8μM ADP
B. 0.08μM adrenaline + RD
C. 0.08μM adrenaline + 0.8μM ADP.
Aggregation tracings are shown for PRP prepared from citrated blood. The arrows indicate the points of addition of the agonists in the order given above. Similar results were obtained with four other donors whose TXB_2 levels were 0.4-2.0 pmol/10^7 platelets for irreversible aggregation. ND = not detected.

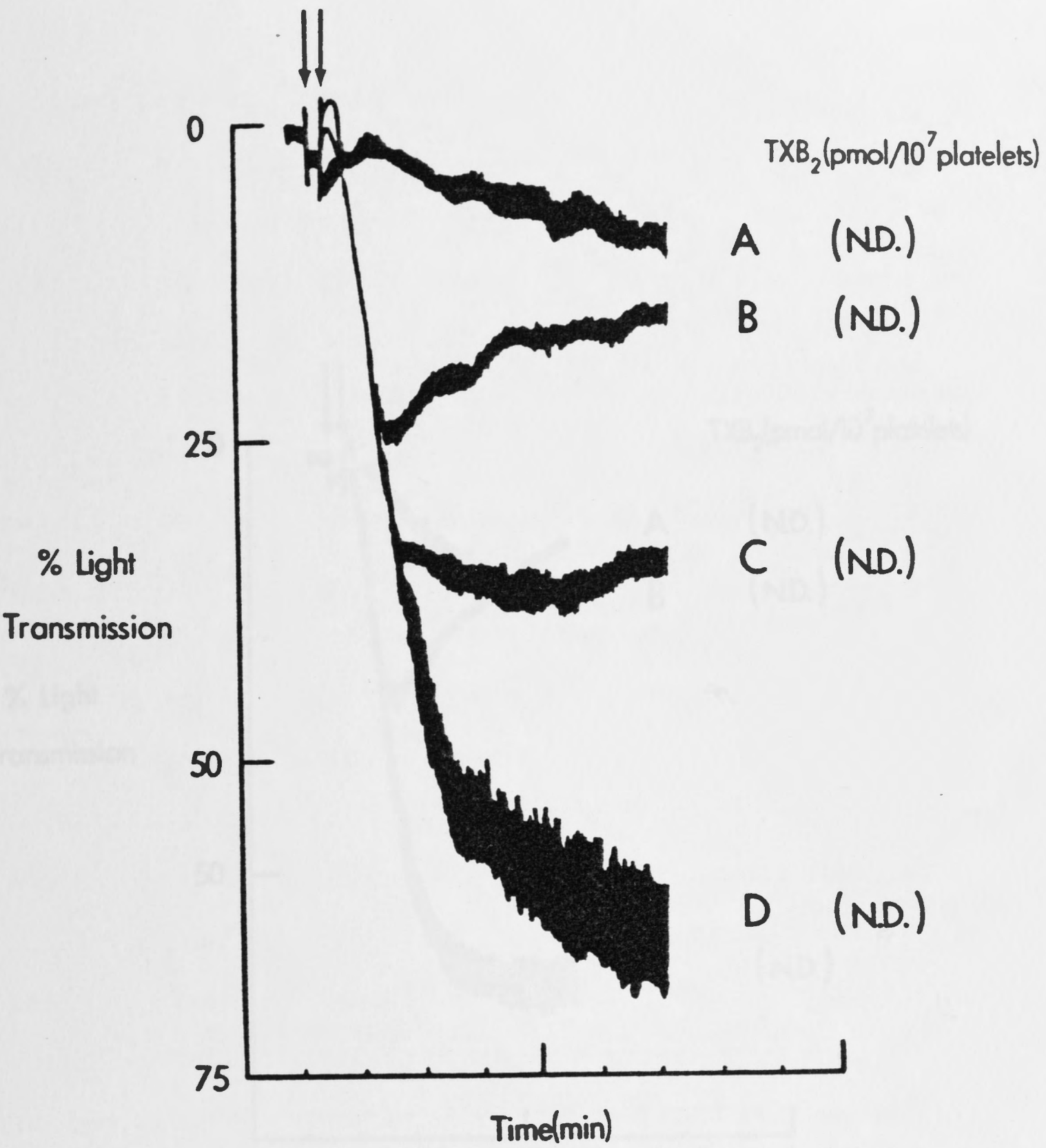

Figure 4.2 The influence of aspirin on the potentiation of ADP-induced aggregation by adrenaline

Aggregation tracings for the responses in the presence of 100μM ASA are shown. The synergistic effect of ADP and adrenaline in the presence of ASA was not associated with the production of TXB_2.

A. 0.08μM adrenaline + RD.
B. RD + 1.6μM ADP.
C. 0.08μM adrenaline + 1.6μM ADP.
D. 0.8μM adrenaline + 1.6μM ADP.

ND = not detected.

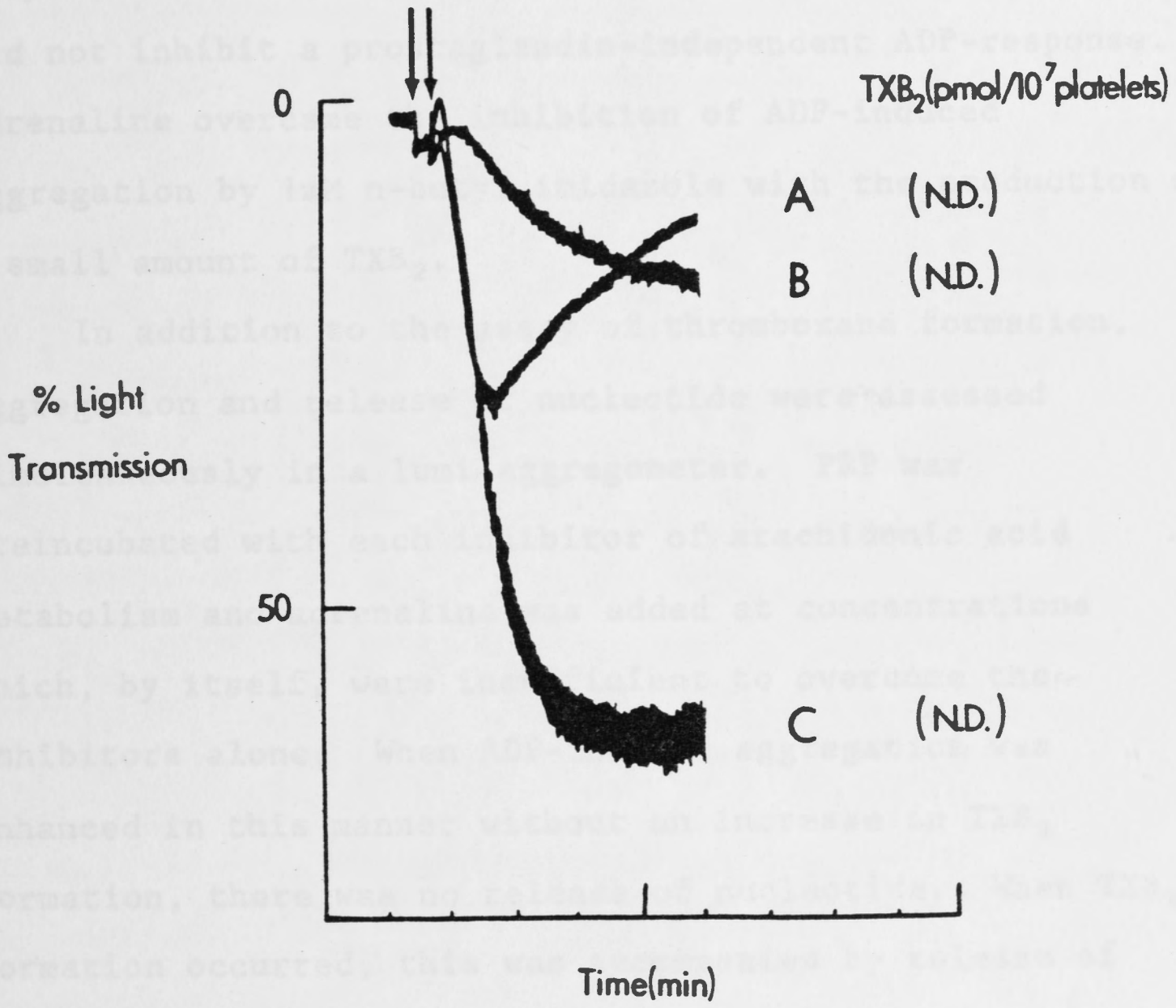

Figure 4.3 The influence of indomethacin on the potentiation of ADP-induced aggregation by adrenaline.

PRP was pre-incubated for 1 min with 100μM indomethacin.
A. RD + 0.8μM ADP.
B. 8μM adrenaline + RD.
C. 8μM adrenaline + 0.8μM ADP.
Similar results were obtained with two other donors using indomethacin concentrations as low as 10μM. ND = not detected.

adrenaline occurred again with no increase in TXB_2 formation. When the concentration of BW755C was decreased to 3.8μM, potentiation occurred with a small increase in TXB_2 formation. The thromboxane synthetase inhibitor, n-butyl imidazole (5mM) also behaved like indomethacin and did not inhibit a prostaglandin-independent ADP-response. Adrenaline overcame the inhibition of ADP-induced aggregation by 1mM n-butyl imidazole with the production of a small amount of TXB_2.

In addition to the assay of thromboxane formation, aggregation and release of nucleotide were assessed simultaneously in a lumi-aggregometer. PRP was preincubated with each inhibitor of arachidonic acid metabolism and adrenaline was added at concentrations which, by itself, were insufficient to overcome the inhibitors alone. When ADP-induced aggregation was enhanced in this manner without an increase in TXB_2 formation, there was no release of nucleotide. When TXB_2 formation occurred, this was accompanied by release of nucleotide.

4.3.2 Potentiation of Potassium Arachidonate (KA)-induced Aggregation

Potentiation by adrenaline of KA-induced aggregation in the absence of inhibitors was associated with an increase in TXB_2 formation (Fig 4.4).

When PRP was preincubated with aspirin (100μM) potentiation of KA-induced aggregation could only be achieved with high(45μM) concentrations of adrenaline(Fig

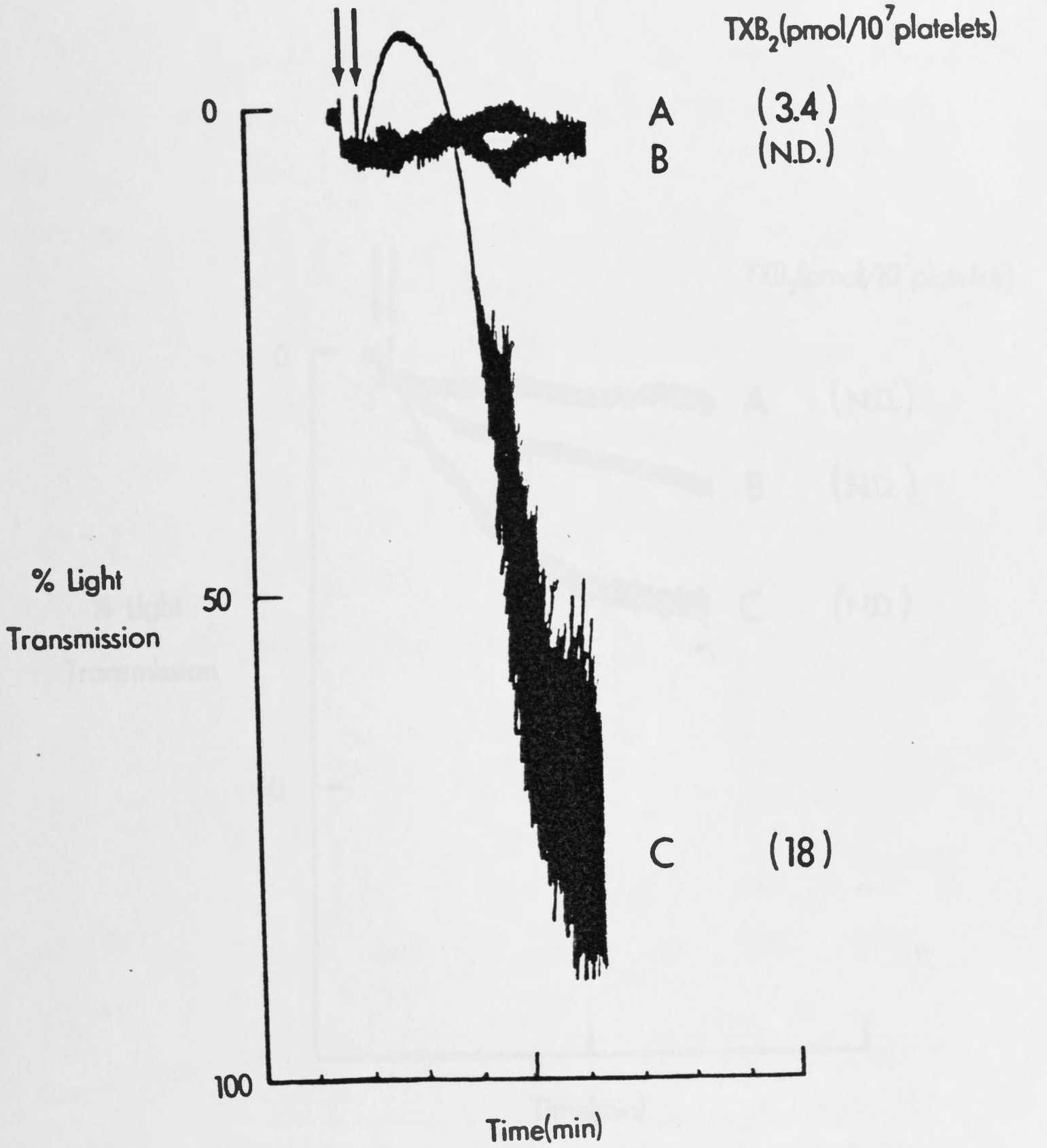

Figure 4.4 The potentiation of KA-induced aggregation by adrenaline

A. RD + 160μM KA.
B. 0.08μM adrenaline + RD.
C. 0.08μM adrenaline + 160μM KA.
In another experiment where 160μM KA was the subthreshold concentration, TXB_2 levels were 1.6 p mol/10^7 platelets for KA only and 6.6 p mol/10^7 platelets for adrenaline-potentiated KA-induced aggregation.
ND = not detected.

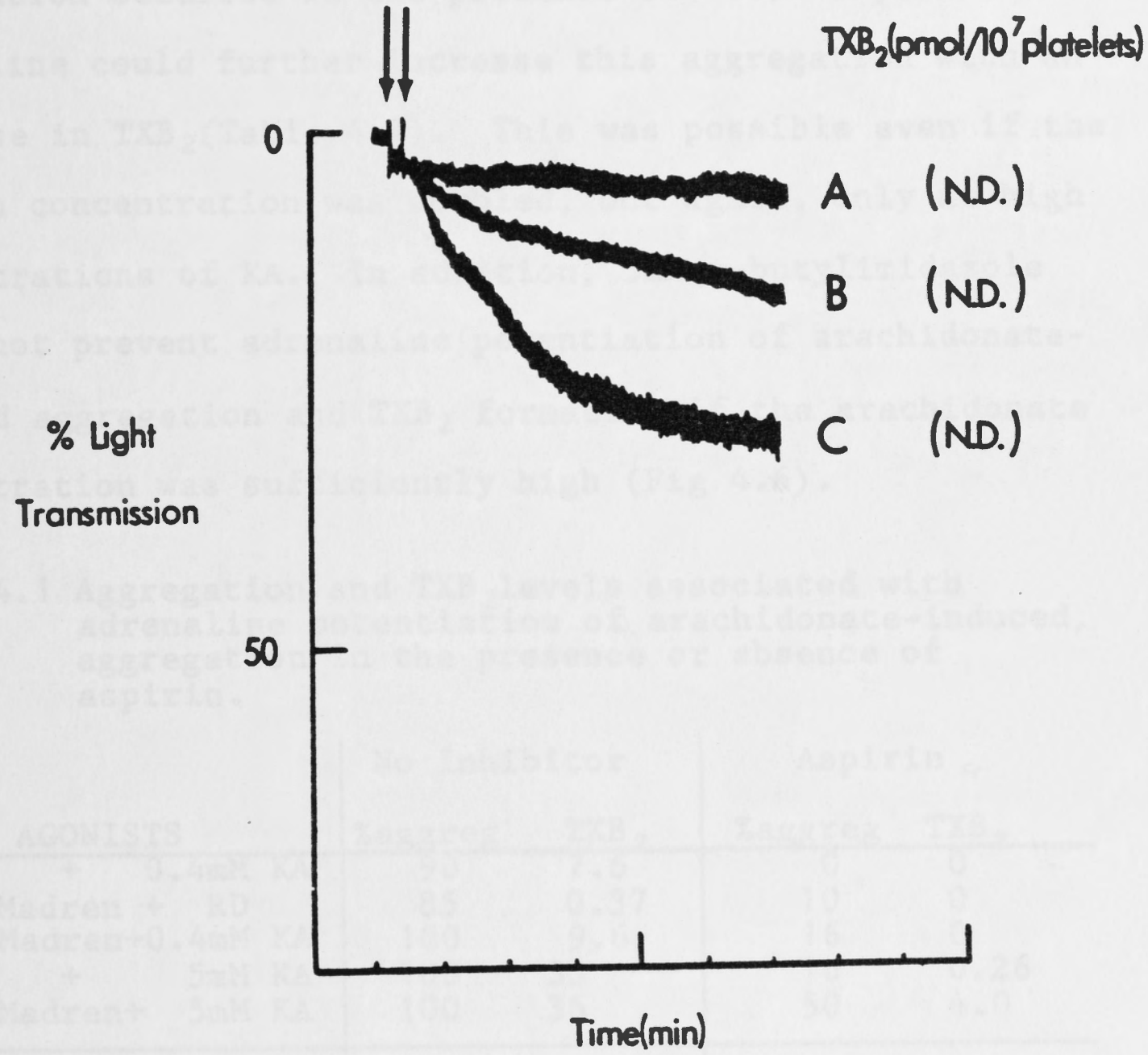

Figure 4.5 The influence of aspirin on the potentiation of KA-induced aggregation by adrenaline.

PRP was preincubated with 100μM ASA as described.
A. RD + 400μM KA.
B. 45μM adrenaline + RD.
C. 45μM adrenaline + 400μM KA.
ND = not detected.

4.5). This potentiation was small and occurred without any increase in TXB_2. If the concentration of KA was increased approximately 10-fold, such that a small amount of aggregation occurred in the presence of 100μM aspirin, adrenaline could further increase this aggregation with an increase in TXB_2(Table 4.1). This was possible even if the aspirin concentration was doubled, but again, only at high concentrations of KA. In addition, 5mM n-butylimidazole could not prevent adrenaline potentiation of arachidonate-induced aggregation and TXB_2 formation if the arachidonate concentration was sufficiently high (Fig 4.6).

Table 4.1 Aggregation and TXB_2 levels associated with adrenaline potentiation of arachidonate-induced aggregation in the presence or absence of aspirin.

	No Inhibitor		Aspirin	
AGONISTS	%aggreg	TXB_2	%aggreg	TXB_2
#1.RD + 0.4mM KA	90	7.6	0	0
45μMadren + RD	85	0.37	10	0
45μMadren+0.4mM KA	100	9.6	16	0
RD + 5mM KA	100	35	10	0.26
45μMadren+ 5mM KA	100	35	50	4.0
#2.RD + 0.4mM KA	0	0.25	0	0
45μMadren + RD	15	0.92	10	0
45μMadren+0.4mM KA	90	29	10	0
RD + 3.6mM KA	100	45	8	2.2
45μMadren+3.6mM KA	100	45	50	7.2

The data for two separate experiments are shown. 100μM aspirin was used for exp#1 and 200μM aspirin was used for exp#2. The abbreviations used are: KA = potassium arachidonate, adren = adrenaline, RD = reagent diluent, TXB_2 = thromboxane B_2(pmol/10^7platelets).

When PRP was preincubated with 10-100μM indomethacin, however, potentiation of KA-induced aggregation by

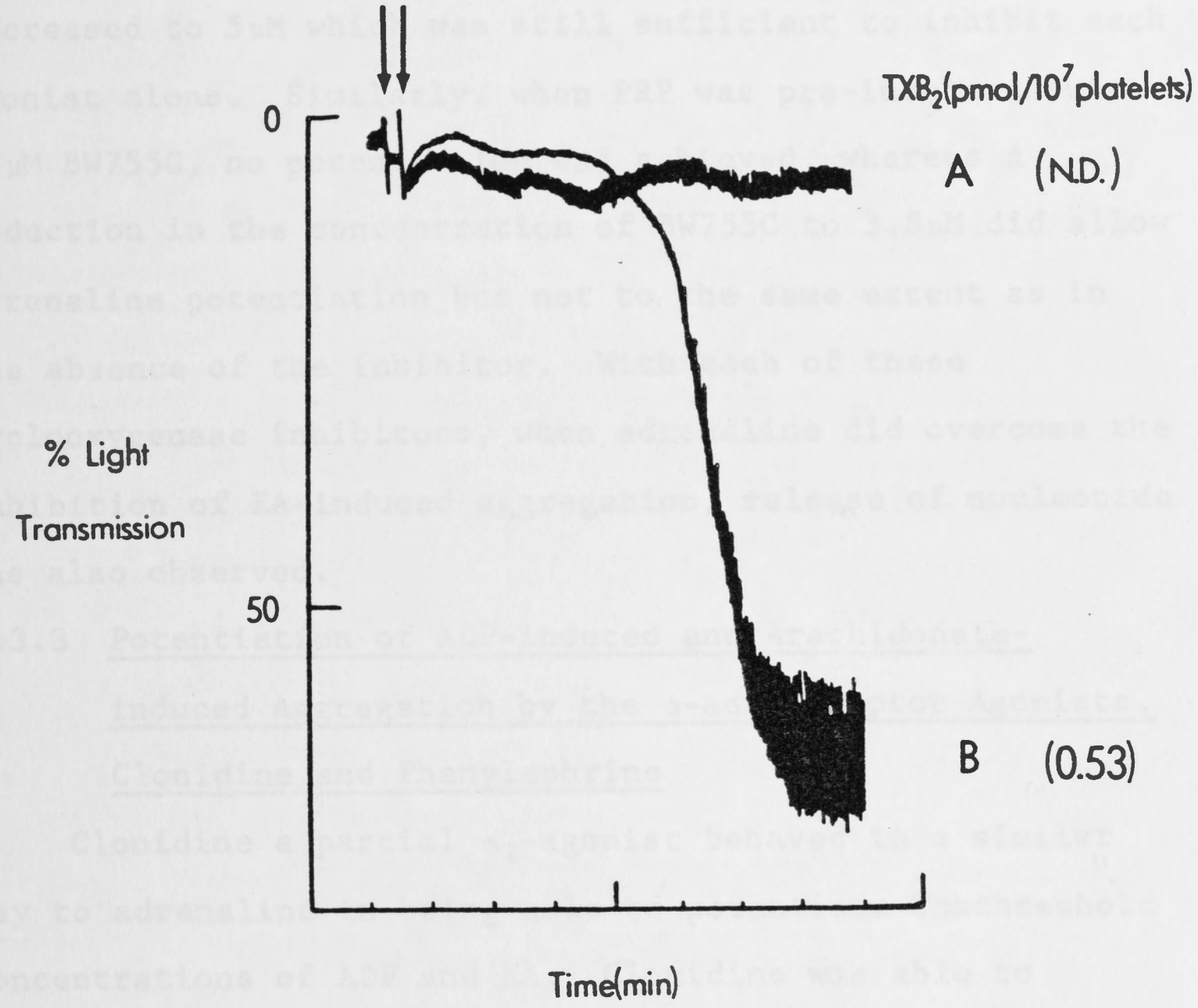

Figure 4.6 The influence of n-butylimidazole on the potentiation of KA-induced aggregation by adrenaline

PRP was preincubated with 5mM n-butylimidazole for 1 min.
A. 0.8μM adrenaline + 400μM KA.
B. 0.8μM adrenaline + 600μM KA.
Each agonist at these concentrations was inhibited when added separately.
ND = not detected.

adrenaline (up to 8μM) was not achieved. Some potentiation by adrenaline was achieved with an increase in TXB_2 formation when the concentration of indomethacin was decreased to 5μM which was still sufficient to inhibit each agonist alone. Similarly, when PRP was pre-incubated with 38μM BW755C, no potentiation was achieved, whereas a reduction in the concentration of BW755C to 3.8μM did allow adrenaline potentiation but not to the same extent as in the absence of the inhibitor. With each of these cyclooxygenase inhibitors, when adrenaline did overcome the inhibition of KA-induced aggregation, release of nucleotide was also observed.

4.3.3 Potentiation of ADP-induced and Arachidonate-induced Aggregation by the α-adrenoceptor Agonists, Clonidine and Phenylephrine

Clonidine a partial α_2-agonist behaved in a similar way to adrenaline in being able to potentiate subthreshold concentrations of ADP and KA. Clonidine was able to potentiate responses to ADP in the presence of the cyclooxygenase inhibitors without TXB_2 formation. Clonidine, therefore, facilitated cyclooxygenase-independent aggregation.

Clonidine usually did not cause aggregation by itself even at concentrations as high as 800μM. However, two patients with thrombotic disorders were found to respond to 0.8μM and 800μM clonidine respectively. The concentrations of clonidine which potentiated aggregation due to ADP in the presence of aspirin were comparable to those of

adrenaline, that is in the range 0.8 - 8μM. Nevertheless, in any one donor, a concentration of clonidine which potentiated an ADP reponse was not as effective as the same concentration of adrenaline (Fig 4.7).

Some donors exhibited potentiation of the ADP response by the α_1-agonist, phenylephrine. Those donors who did, also exhibited an additive response to the combination of clonidine and phenylephrine (Fig 4.7). Much higher concentrations of phenylephrine than of clonidine were required to potentiate ADP. For the donor illustrated in Fig 4.7, 8.0μM phenylephrine potentiated 0.4μM ADP with an associated TXB_2 production of 0.46 pmole/10^7platelets (2.4ng/ml).

4.3.4 Post-Receptor Inhibitors

As discussed previously, there is some evidence that dibenamine and phenoxybenzamine may be acting on the stimulus-response coupling sequence distal to the receptor. These drugs were therefore used to probe post-receptor events. Both dibenamine (0.2mM) and phenoxybenzamine (0.2mM) inhibited the aggregation response of platelets to adrenaline even when the concentration of adrenaline was increased to 80μM. Adrenaline induced TXB_2 formation was also inhibited by these drugs. These drugs also prevented the second phase of the ADP response with a reduction of the first phase, but had no effect on the ADP-induced shape change. Inhibition of the second phase of the ADP response was associated with an absence of TXB_2 formation.

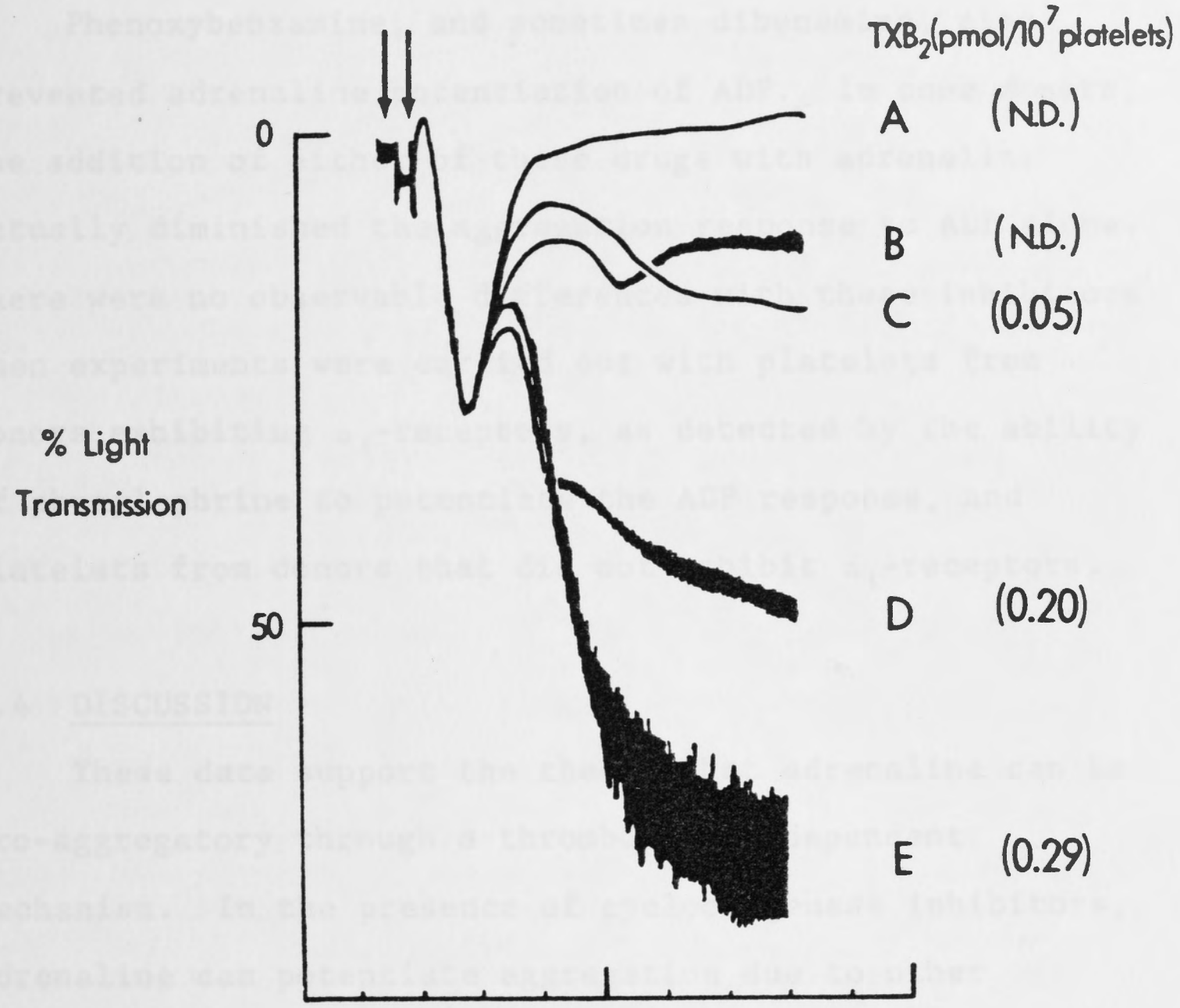

Figure 4.7 Potentiation of ADP-induced aggregation by adrenaline, clonidine and phenylephrine.

The concentration of ADP for all tracings was 0.4μM. The additional agonists (added at the first arrow) were:
A. RD.
B. 0.8μM phenylephrine.
C. 0.008μM clonidine.
D. 0.008μM adrenaline.
E. 0.8μM phenylephrine + 0.008μM clonidine.
This donor was one of a small percentage of donors whose ADP response was potentiated by phenylephrine in addition to clonidine. This donor is extremely sensitive to adrenaline as indicated by the very low concentration (0.008μM) which potentiated the ADP response.
ND = not detected.

Phenoxybenzamine, and sometimes dibenamine, also prevented adrenaline potentiation of ADP. In some donors, the addition of either of these drugs with adrenaline actually diminished the aggregation response to ADP alone. There were no observable differences with these inhibitors when experiments were carried out with platelets from donors exhibiting α_1-receptors, as detected by the ability of phenylephrine to potentiate the ADP response, and platelets from donors that did not exhibit α_1-receptors.

4.4 DISCUSSION

These data support the theory that adrenaline can be pro-aggregatory through a thromboxane-independent mechanism. In the presence of cyclooxygenase inhibitors, adrenaline can potentiate aggregation due to other agonists. Adrenaline can also increase thromboxane production despite the presence of aspirin, when high concentrations of arachidonate are added. In the absence of cyclooxygenase inhibitors, adrenaline probably acts through both mechanisms and these are expected to involve activation of α_2-receptors which is supported by the potentiating effects of clonidine described in this chapter.

Grant and Scrutton(1979) identified the α_2-adrenoceptor as being primarily responsible for mediating the response to adrenaline. They showed that the potentiation of ADP-induced aggregation by clonidine was inhibitable by yohimbine, a selective α_2-antagonist. In

contrast, indoramin, a selective α_1-antagonist, was relatively ineffectual as an inhibitor of clonidine-stimulated responses. Inhibition of responses to clonidine by yohimbine occurred over a range of concentrations significantly higher than that required for inhibition of adrenaline-induced responses. Clonidine is therefore regarded as a partial α_2-agonist. Since the possible α_1-receptor is not exhibited on platelets from all donors as shown by methoxamine potentiation and specific [^{3}H]-prazosin binding (Scrutton,1982), it cannot be the predominant receptor involved in activation of platelets by adrenaline.

Some conversion of arachidonate to TXB_2 is possible despite irreversible acetylation of cyclooxygenase by aspirin if sufficiently large amounts of exogenous arachidonate are added. Whatever the mechanism of this reaction, high concentrations of adrenaline can enhance it. Concentrations of indomethacin and BW755C less than that of aspirin, inhibited the cyclooxygenase pathway no matter how much adrenaline was added. The chemical nature of the interference of the inhibitor with the enzyme active site may determine whether adrenaline can increase cyclooxygenase activity (Humes et al., 1981). Another explanation for the greater effectiveness of indomethacin may be that this inhibitor also reduces the amount of available endogenous arachidonate (Erman et al., 1980).

Indomethacin was effective in preventing adrenaline potentiation of KA-induced aggregation but not when the inhibitor concentration was reduced to 5μM. Similarly, BW755C, which inhibits both cyclooxygenase and

lipoxygenase, prevented adrenaline potentiation of KA-induced aggregation at a concentration of 38μM, but not at one-tenth this concentration. Indomethacin(100μM) inhibits lipoxygenase by 20% (Higgs et al.,1979) and can therefore also be a dual inhibitor like BW755C. It is possible that adrenaline achieves potentiation by overcoming inhibition of the lipoxygenase pathway of arachidonate acid metabolism, although no role has yet been assigned to the products of the lipoxygenase pathway in platelet activation.

ADP-induced aggregation can still be enhanced by adrenaline or clonidine under the influence of these inhibitors at concentrations which completely prevent thromboxane production. Adrenaline promotes this thromboxane-independent pathway of ADP-induced aggregation without causing release of nucleotide. Higher concentrations of adrenaline were required to achieve the same extent of aggregation as in the absence of thromboxane inhibitors and much higher concentrations than found in vivo. Without the amplifying effect of thromboxanes and nucleotide, a larger stimulus seems to be required to achieve platelet activation by the thromboxane-independent pathway.

This thromboxane-independent aggregation is also irreversible and can appear indistinguishable from the pattern of irreversible aggregation which is accompanied by release, discerned from recorder tracings only. Additional information on thromboxane formation and the release

reaction are needed to characterize the aggregation response in more detail. What is usually referred to as first phase aggregation could also be referred to as cyclooxygenase-independent aggregation.

There could be several mechanisms by which adrenaline promotes thromboxane-independent aggregation. It has recently been proposed that adrenaline by binding to α-receptors, enhances calcium (Ca^{2+}) mobilization and thereby promotes aggregation (Rao et al., 1981a). Stimulation of the α-receptor also increases Ca^{2+} uptake (Owen et al., 1980) but further work (Owen and Le Breton, 1980) showed that potentiation of the ADP response by subthreshold levels of adrenaline was not associated with measurable Ca^{2+} uptake. Calcium redistribution was considered to be involved since the Ca^{2+} channel blocker, verapamil, inhibited potentiation, but it is now acknowledged that verapamil achieves an inhibitory effect at the level of the^{+} α-adrenergic receptor (Barnathan et al.,1982). Brass and Shattil(1982) were unable to find an enhancement of net Ca^{2} uptake by ADP or adrenaline although both these agonists increased the extent of binding of Ca^{2+} to the surface and also the rate of exchange into the intracellular pool. The biochemical changes leading to this possible redistribution. of Ca^{2+} are not understood and probably only some of the many effects of Ca^{2+} in platelets have been so far revealed Inositol-1,4,5-trisphosphate is a possible mediator of calcium redistribution(Berridge,1984).

α_2-receptor stimulation is thought to be universally associated with inhibition of adenylate cyclase (Fain and Garcia-Sainz, 1980), reducing c'AMP production which could be related to calcium redistribution (Kaser-Glanzman et al., 1977). Rao et al. (1981a) reported, however, that adrenaline can potentiate platelet responses without measurable changes in c'AMP levels. No net decrease in c'AMP results on exposure of resting platelets to adrenaline (Haslam,1978a,b). Furthermore, the induction of aggregation by adrenaline has been shown to be dissociated from its effect as an inhibitor of adenylate cyclase, by removal of extracellular Na^+ (Connolly and Limbird,1983) or by the use of certain antagonists (Clare et al., 1984).

There could also be other membrane changes in response to α-receptor stimulation. For other tissues, besides platelets, it has been suggested that α_1-receptors mediate phosphatidylinositol (PI) turnover and hence the release of bound intracellular Ca^{2+}, as well as the entry of extracellular Ca^{2+} (Fain and Garcia-Sainz, 1980; Jafferji and Michell, 1976). These Ca^{2+} responses are precisely the reported effects of adrenaline on platelets. The effects of adrenaline on platelets, however, are believed to be mediated by α_2-receptors, not α_1-receptors (Grant and Scrutton, 1979). It is also not known whether adrenaline, while potentiating ADP, affects PI turnover. Very recently, Clare and Scrutton(1984) failed to show PI breakdown due to adrenaline alone in the absence of

secretion. In either case polyphosphoinositides have not been examined.

The fact that phenoxybenzamine totally inhibits the adrenaline response in the platelet is consistent with PI turnover being important in mediating the adrenergic effects. The results are obviously difficult to interpret, however, since phenoxybenzamine is an α-receptor($\alpha_1$greater thanα_2)antagonist in addition to possessing post-receptor inhibitory activity (Jafferji and Michell, 1976). There is no need to necessarily assume that PI breakdown would depend on α_1-receptor activation since the particular effects associated with a particular receptor demonstrated in other tissues may not apply to the platelet. The effects of phenoxybenzamine on adrenaline stimulation and potentiation require further study, possibly in association with analysis of membrane phospholipids.

The inhibition by dibenamine is consistent with Ca^{2+} mobilization being a prerequisite for expression of adrenaline stimulation of α-receptors. Even though there is some evidence that calcium uptake may not to be involved in the mechanism of adrenaline activation, calcium movement within the membrane may still be involved. It is possible that effects on calcium may be independent of the PI effect, since dibenamine seems to act independently of PI turnover. The present findings concur with those of Rao *et al.*(1980a) in establishing the existence of a thromboxane-independent pathway of adrenaline potentiation and both studies show the ability of adrenaline to influence the

aggregation response of aspirin-treated platelets to arachidonate. Adrenaline, together with higher concentrations of arachidonate, partially restores the activity of cyclooxygenase, under conditions appropriate for total enzyme inhibition.

Following cyclooxygenase inhibition, much higher concentrations of adrenaline are required to achieve the same potentiation of ADP-induced aggregation as that observed when the thromboxane pathway is operating. It is unlikely that such concentrations would ever be achieved in the circulation, and therefore thromboxane formation is probably essential for the complete pro-aggregatory effect of adrenaline *in vivo*. The influence of other agonists present in the circulation on the pro-aggregatory effect of adrenaline has been shown(Chapter 3). Therefore the effective concentration of adrenaline with or without cyclooxygenase inhibition, *in vivo* is expected to be much lower than the concentrations used *in vitro*. A thromboxane-independent pathway is involved in adrenaline potentiation the mechanism of which remains unresolved.

CHAPTER 5

THE INFLUENCE OF PHOSPHOLIPASE A_2 INHIBITORS ON ADRENALINE POTENTIATION

5.0 SUMMARY

To further characterize adrenaline potentiation of ADP-induced aggregation, the influence of phospholipase A_2 inhibitors was examined. The inhibitors chosen were bromophenacyl bromide (BPB), a widely reported inhibitor of phospholipase A_2; tosylphenylalaninechloromethyl ketone (TPCK), a chymotrypsin inhibitor with reported phospholipase A_2 inhibitory properties; and phenothiazines which inhibit several enzymes including calmodulin-dependent enzymes and protein kinase C.

BPB and TPCK shared in common the ability to enhance thromboxane production when adrenaline potentiated the ADP-response. Adrenaline therefore enhances arachidonic acid release by a mechanism independent of phospholipase A_2 activity in the presence of these two inhibitors. The phenothiazines, chlorpromazine and trifluoperazine inhibited thromboxane production but adrenaline potentiation of thromboxane-independent aggregation was still observed.

It is suggested that adrenaline promotes diacylglycerol production from which arachidonic acid release is promoted via lipase activity, in the presence of BPB or TPCK, but not in the presence of phenothiazines, in which case the kinase may be more active.

5.1 INTRODUCTION

In the previous chapter, the mechanism of adrenaline potentiation of ADP and arachidonate-induced aggregation was examined. While adrenaline was found to stimulate a non-prostaglandin pathway there was also evidence of some stimulation of arachidonic acid metabolism. Perhaps adrenaline has a direct effect on cyclooxygenase or thromboxane synthetase activity. It was therefore decided to inhibit the prostaglandin pathway by inhibition of arachidonate release because it was thought that inhibition of arachidonate release might allow further characterization of the effects of adrenaline leading to both prostaglandin-dependent and independent pathways of activation.

As discussed in Chapter 1, there are several ways in which arachidonate might be released from membrane phospholipids(Fig 5.1). Phospholipase A_2 enzymes could release the fatty acid from phosphatidic acid or otherwise from the phospholipids, phosphatidylcholine, phosphatidylethanolamine, phosphatidylserine or phosphatidylinositol(PI) (Billah *et al*., 1980; 1981). Alternatively, phospholipase C and diacylglycerol lipase activity can lead to the release of arachidonic acid from PI (Bell *et al*., 1979). The respective contributions of these different phospholipases to arachidonic acid release has not been resolved.

It has been difficult to find specific inhibitors of either phospholipase A_2 or phospholipase C. Vallee *et al*.

POSSIBLE SOURCES OF ARACHIDONIC ACID

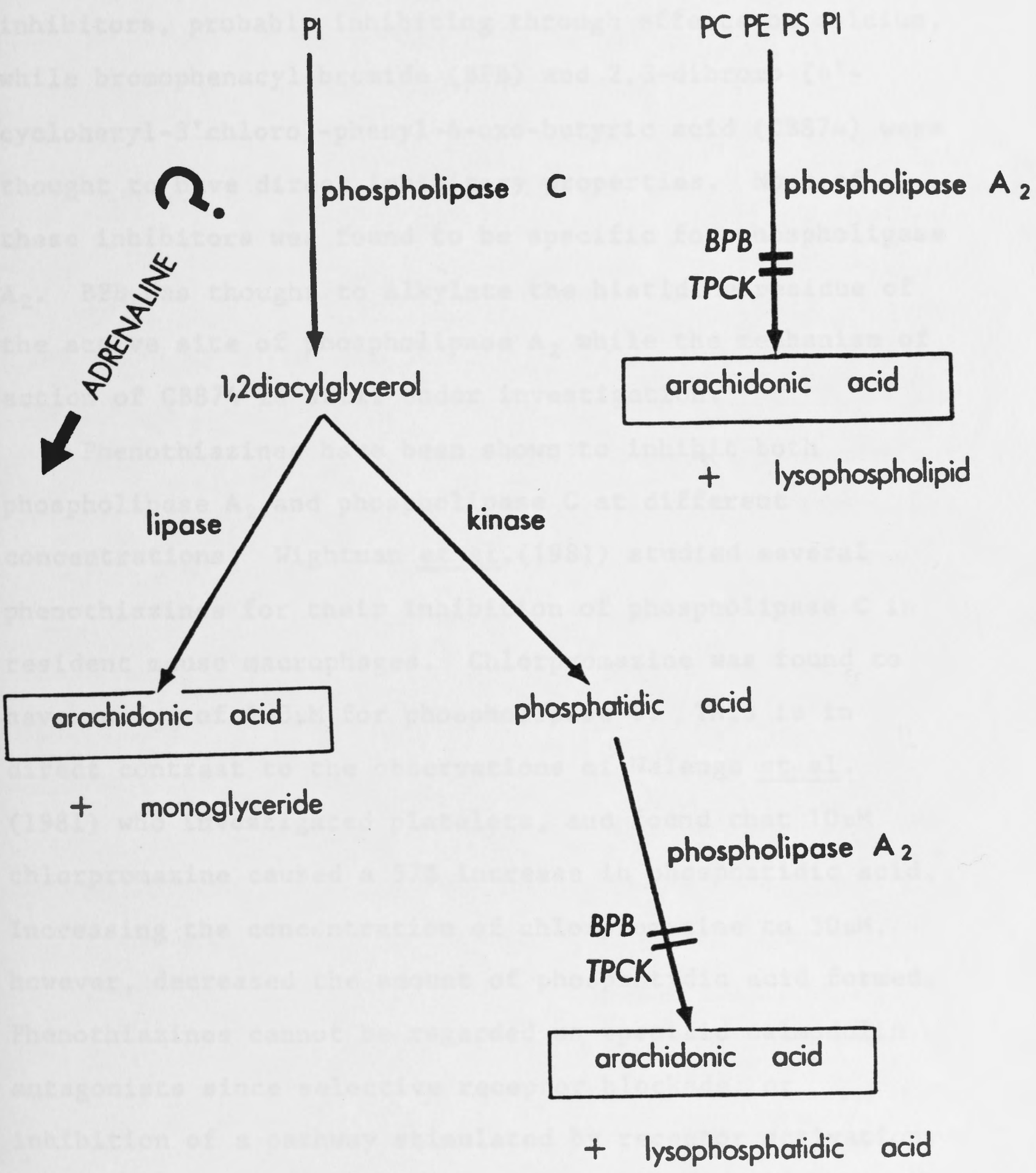

Figure 5.1 The release of arachidonic acid from platelet phospholipids.

A simplified diagram of the reactions and enzymes involved in the possible mechanisms of arachidonate release. The proposed influence of adrenaline under conditions of potentiation of ADP-induced aggregation on these reactions is shown.

(1979) assessed four different phospholipase A_2 inhibitors. Mepacrine and papaverine were found to be indirect inhibitors, probably inhibiting through effects on calcium, while bromophenacyl bromide (BPB) and 2,3-dibromo (4'-cyclohexyl-3'chloro)-phenyl-4-oxo-butyric acid (CB874) were thought to have direct inhibitory properties. None of these inhibitors was found to be specific for phospholipase A_2. BPB was thought to alkylate the histidine residue of the active site of phospholipase A_2 while the mechanism of action of CB874 is still under investigation.

Phenothiazines have been shown to inhibit both phospholipase A_2 and phospholipase C at different concentrations. Wightman et al.(1981) studied several phenothiazines for their inhibition of phospholipase C in resident mouse macrophages. Chlorpromazine was found to have an I_{50} of 6.3μM for phospholipase C. This is in direct contrast to the observations of Walenga et al. (1981) who investigated platelets, and found that 10μM chlorpromazine caused a 57% increase in phosphatidic acid. Increasing the concentration of chlorpromazine to 30μM, however, decreased the amount of phosphatidic acid formed. Phenothiazines cannot be regarded as specific calmodulin antagonists since selective receptor blockade, or inhibition of a pathway stimulated by receptor activation, has also been shown by Cocks et al. (1981) for liver and smooth muscle. Chlorpromazine has been shown to inhibit protein kinase C in some circumstances(Mori et al.,1980). Lullman et al. (1981) reported that phenothiazines can

displace bound Ca^{2+} from PI monolayers at an I_{50} of approximately 50μM. Phospholipase C activity requires calcium (Billah et al.,1980) and at certain concentrations, phenothiazines provide calcium for phospholipase C in the cytosol. Receptor affinity and calcium availability could determine whether phenothiazines behave as inhibitors or stimulators.

BPB and tosylphenylalanine-chloromethylketone (TPCK) were shown by Vargaftig et al. (1980b) to inhibit aggregation caused by thrombin or ionophore A23187. Both were shown to inhibit phospholipase A_2 activity. BPB was not specific for phospholipase A_2 since it also reduced the yield of thromboxane A_2 from exogenously added arachidonic acid, indicating an inhibition of cyclooxygenase or synthetase. Short incubation times, however, showed a preferential inhibition of phospholipase A_2 over cyclooxygenase or thromboxane synthetase. TPCK caused a reduction in thromboxane release but this was shown to not be significant. BPB can also inhibit acetyl and acyltransferases which could affect the synthesis of platelet activating factor (PAF) (Wykle et al., 1980). BPB can also presumably inhibit the putative acyltransferase enabling mobilization of arachidonic acid between membrane phospholipids (Billah et al., 1980). It has also been shown with crude platelet extracts that BPB is an inhibitor of the PI-specific phospholipase C-diglyceride lipase pathway (Hofmann et al., 1982). Another cautionary note in the use of BPB comes from the work of Smolen and Weissman

(1980) who showed that BPB also inhibits superoxide anion generation in human polymorphonuclear leucocytes, a normal response to surface stimuli. What effect such inhibition may have in the platelet is unknown.

Serine esterase inhibitors have been shown to inhibit PI and phosphatidylcholine (PC) metabolism (Walenga et al., 1980). These authors concluded that serine esterase inhibitors are therefore inhibitors of phospholipase C and possibly, of phospholipase A_2. Their evidence for inhibition of PI turnover induced by A23187 could actually be due to inhibition of phospholipase A_2. It has subsequently been shown by Rittenhouse-Simmons (1981) that the action of phospholipase A_2 is responsible for the release of arachidonic acid from PI by A23187. Serine esterase inhibitors therefore appear to be inhibitors of both phospholipase C and A_2.

In this chapter the effects of BPB, TPCK and phenothiazines on adrenaline potentiation of ADP is examined.

5.2 METHODS AND MATERIALS

Blood collection in citrate, platelet aggregation and thromboxane B_2 assay were carried out as before.

Bromophenacyl bromide (Koch Light) and tosylphenyl-alaninechloromethylketone (Serval) were dissolved in dimethylsulphoxide (DMSO) and added at one-hundredth of the plasma volume. The phenothiazines, clorpromazine and

trifluoperazine (Smith, Kline and French) were dissolved in reagent diluent (RD).

5.3 RESULTS

5.3.1 Potentiation of the ADP-response by Adrenaline in the Presence of Bromophenacyl Bromide (BPB).

BPB(10μM) completely inhibited the response to 8μM adrenaline which would otherwise cause irreversible aggregation. BPB also inhibited thromboxane B_2 formation caused by adrenaline. BPB did not completely inhibit the response to threshold ADP, but inhibited the second phase along with TXB_2 production and also reduced the first phase. BPB did not affect the shape change due to ADP. BPB also inhibited the response to threshold exogenous arachidonate, reducing but not totally inhibiting thromboxane formation. Table 5.1 summarizes an experiment exemplifying these properties of BPB. The amount of TXB_2 formed when the arachidonate response was inhibited by BPB was still in excess of that produced in response to adrenaline.

Table 5.1 The ability of bromophenacyl bromide to inhibit aggregation due to adrenaline, ADP and potassium arachidonate.

Agonist	Inhibitor	TXB_2	Aggregation
Adrenaline(8μM)	DMSO	1.3	irreversible
" "	BPB(10μM)	0	no aggregation
ADP(2.5μM)	DMSO	0.3	biphasic shape change and small first phase
" "	BPB(10μM)	0	
Arachidonate(0.12μM)	DMSO	11	complete
" "	BPB(10μM)	1.9	no aggregation

The inhibitor was added 1min prior to each agonist. TXB_2 levels are expressed as pmol/10^7platelets.
DMSO = dimethylsulphoxide(1%).

Despite these inhibitory effects on single agonists, BPB (10μM) did not inhibit the ability of adrenaline to potentiate ADP or arachidonate. Indeed, when potentiation occurred in the presence of BPB, thromboxane formation was increased (Fig 5.2). Not every donor exhibited the dramatic reduction in the first phase of aggregation in the presence of BPB as shown in Fig. 5.2. In all donors, however, there was a 2-3fold increase in TXB_2. BPB (100μM) prevented adrenaline potentiation of the ADP response, as did 50μM BPB for some donors, but 20μM BPB did not inhibit potentiation nor TXB_2 formation.

A time-course experiment revealed that the increase in TXB_2 in the presence of BPB commenced sooner than in the

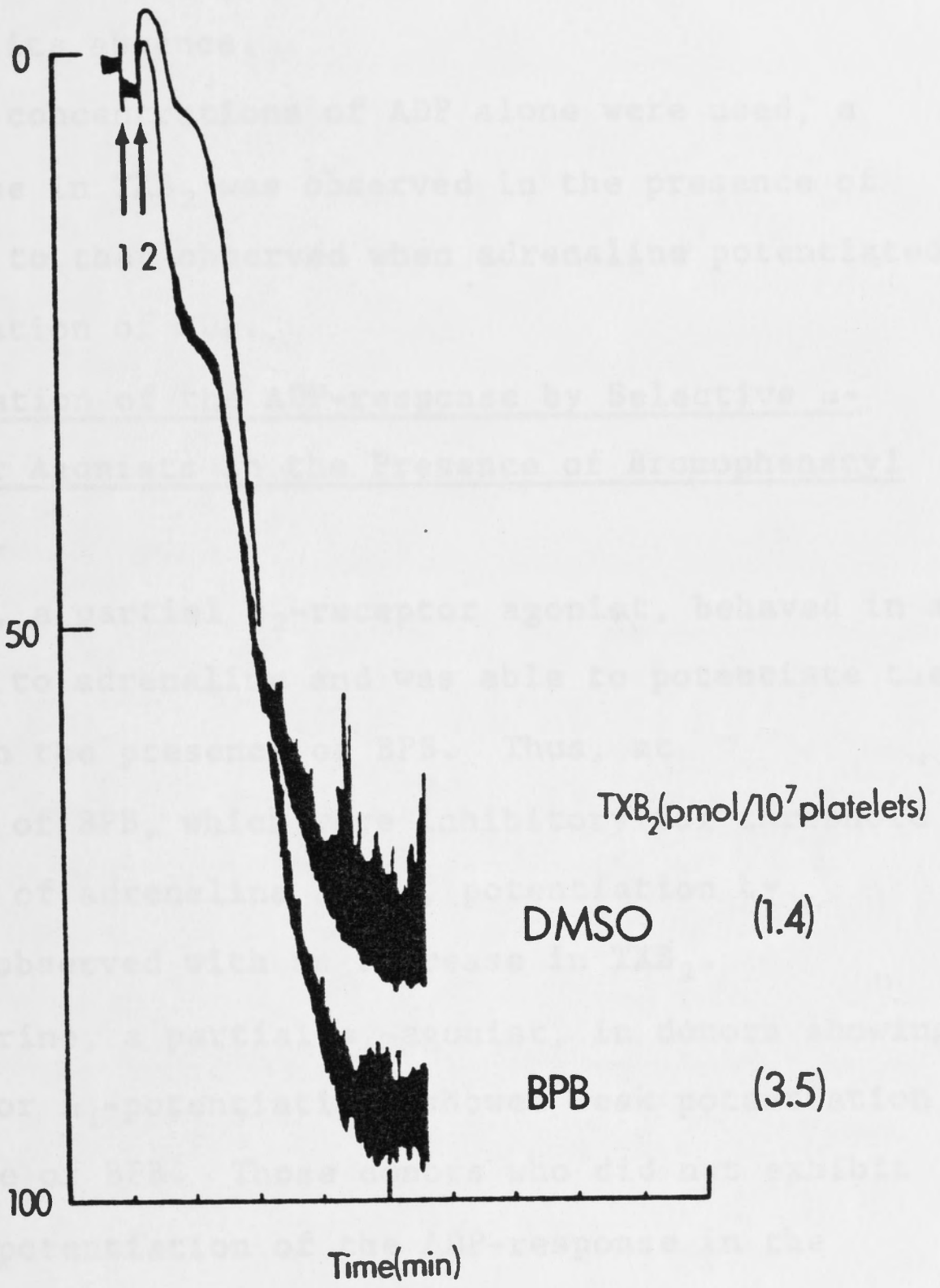

Figure 5.2 Potentiation of the response to ADP by adrenaline in the presence of bromophenacyl bromide (BPB).

The agonist concentrations were
1. 0.8μM adrenaline and
2. 0.8μM ADP.
The PRP was prepared from blood anticoagulated with 10mM citrate and the platelet count was 516,000/μl. BPB or DMSO 1% was added 1 min. before the first agonist, at one-hundredth volume to achieve a concentration of 10μM.

absence of this inhibitor. There was measurable TXB_2 during the first phase of aggregation in the presence of BPB but not in its absence.

When high concentrations of ADP alone were used, a similar increase in TXB_2 was observed in the presence of BPB comparable to that observed when adrenaline potentiated a low concentration of ADP.

5.3.2 Potentiation of the ADP-response by Selective α-receptor Agonists in the Presence of Bromophenacyl Bromide.

Clonidine, a partial α_2-receptor agonist, behaved in a similar manner to adrenaline and was able to potentiate the ADP-response in the presence of BPB. Thus, at concentrations of BPB, which were inhibitory for threshold concentrations of adrenaline alone, potentiation by clonidine was observed with an increase in TXB_2.

Phenylephrine, a partial α_1-agonist, in donors showing the capacity for α_1-potentiation, showed weak potentiation in the presence of BPB. Those donors who did not exhibit phenylephrine potentiation of the ADP-response in the absence of BPB, showed no potentiation in the presence of BPB. Adrenaline was able to potentiate TXB_2 formation in the presence of BPB in donors irrespective of platelet α_1-receptor status.

5.3.3 A Comparison of the Effects of Tosylphenylalanine-chloromethylketone (TPCK) and Bromophenacyl Bromide (BPB) on Adrenaline Potentiation.

TPCK was also tested as an inhibitor of adrenaline

poentiation. TPCK was found to behave in a similar manner to BPB in that increased concentrations of TXB_2 were produced when adrenaline potentiated ADP in the presence of the inhibitor. The result differed in that, at the same concentrations of inhibitors in the same donor, more TXB_2 was produced in the presence of TPCK than BPB (Fig 5.3). Comparable amounts of TXB_2 were produced in the presence of 20μM TPCK or 50μM BPB (in donors in whom this was not inhibitory). It can be seen that the extent of aggregation was not altered very much by these inhibitors at these concentrations, but the first phase of aggregation was decreased and this was associated with earlier TXB_2 formation.

As with BPB, increased TXB_2 production with high concentrations of ADP alone was observed in the presence of TPCK.

5.3.4 Influence of Phenothiazines on the Potentiation of the ADP-response by Adrenaline.

The phenothiazines, chlorpromazine, and trifluoperazine were examined for their influence on adrenaline potentiation. These phenothiazines inhibited thromboxane production due to ADP in a dose-dependent fashion. Adrenaline potentiation of the ADP-response occurred with very little increase in TXB_2 formation in the presence of these drugs; chlorpromazine did not inhibit adrenaline potentiation of the thromboxane-independent pathway (Fig 5.4). Aggregation by adrenaline alone at concentrations which potentiated ADP was completely

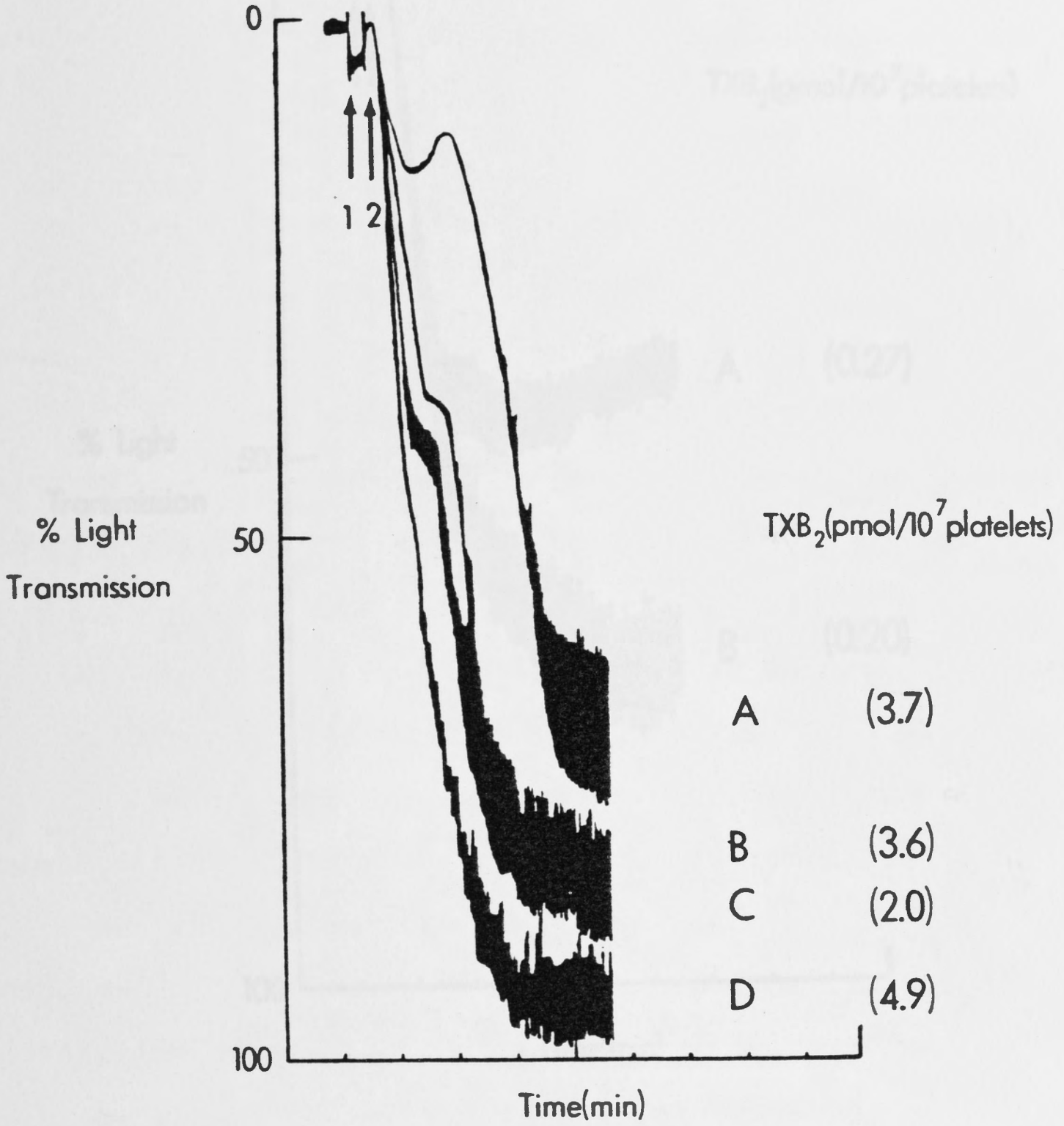

Figure 5.3 Potentiation of the response to ADP by adrenaline in the presence of BPB or TPCK.

The agonist concentrations were
1. 0.8μM adrenaline and
2. 0.8μMADP.
The PRP was prepared from blood anticoagulated with 10mM citrate and the platelet count was 448,000/μl. Similar results were obtained with five other donors. The inhibitor concentrations were
A 50μM BPB
B 20μM TPCK
C DMSO1%
D 50μM TPCK.

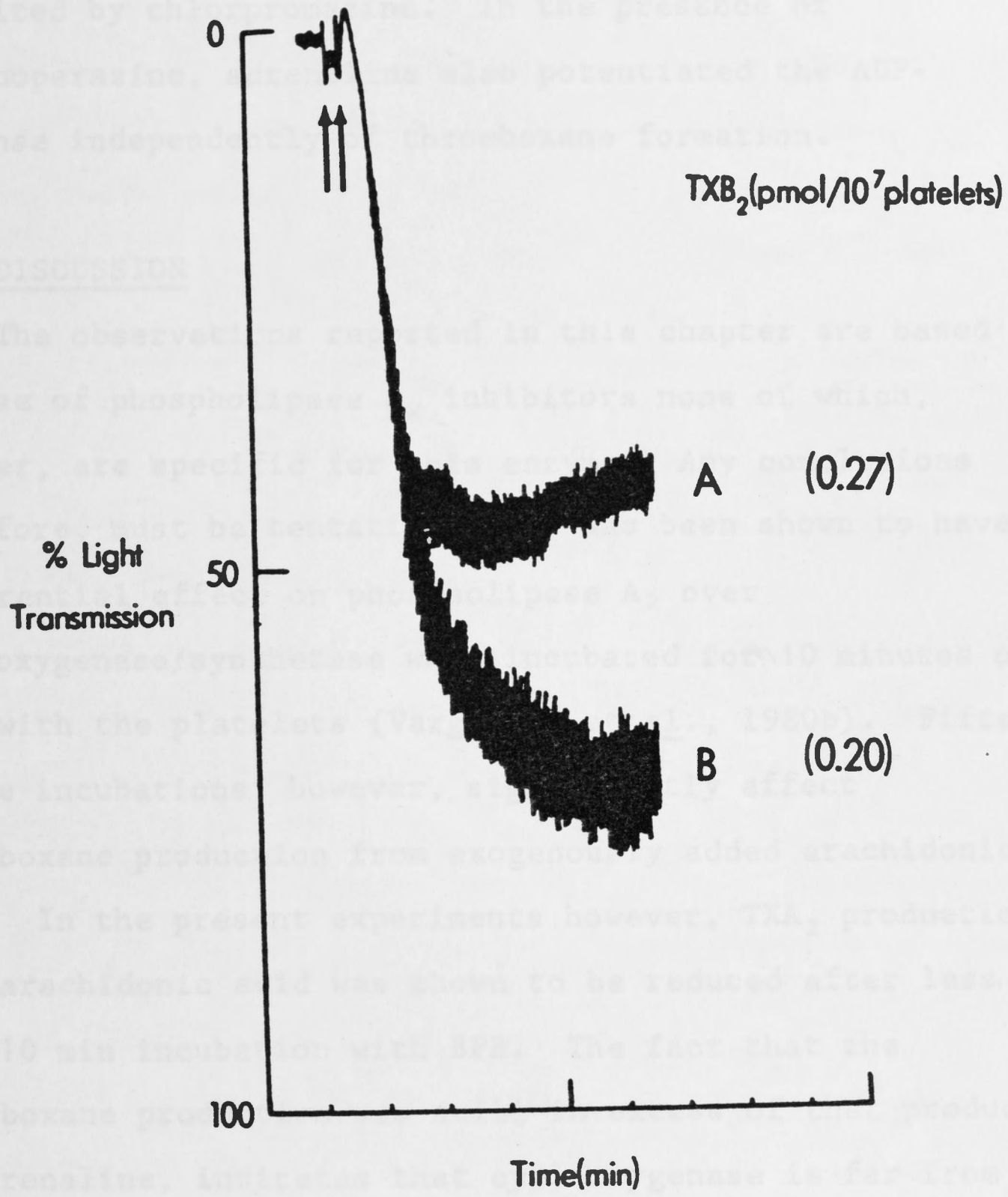

Figure 5.4 Influence of chlorpromazine (CPZ) on adrenaline potentiation of the ADP-response.

The agonist concentrations were
A. RD + 4μMADP.
B. 0.8μMadrenaline + 4μMADP.
The platelets were treated with 50μMCPZ for 1min prior to the addition of the agonists. The PRP was anticoagulated with 10mM citrate and the platelet count was 322,000/μl. In the absence of CPZ the adrenaline potentiated response produced 3.8pmol $TXB_2/10^7$ platelets.

inhibited by chlorpromazine. In the presence of trifluoperazine, adrenaline also potentiated the ADP-response independently of thromboxane formation.

5.4 DISCUSSION

The observations reported in this chapter are based on the use of phospholipase A_2 inhibitors none of which, however, are specific for this enzyme. Any conclusions therefore, must be tentative. BPB has been shown to have a preferential effect on phospholipase A_2 over cyclooxygenase/synthetase when incubated for 10 minutes or less with the platelets (Vargaftig *et al*., 1980b). Fifteen minute incubations, however, significantly affect thromboxane production from exogenously added arachidonic acid. In the present experiments however, TXA_2 production from arachidonic acid was shown to be reduced after less than 10 min incubation with BPB. The fact that the thromboxane production was still in excess of that produced by adrenaline, indicates that cyclooxygenase is far from completely inhibited by BPB and the reduction may be due to other effects of BPB. It is noteworthy also that despite this amount of TXA_2 produced, aggregation was completely inhibited by BPB.

It is not known how preferential BPB might be for phospholipase A_2 over other membrane effects at these incubation times. Hofmann *et al*. (1982) reported that BPB inhibits both PI-specific phospholipase C and diglyceride lipase. Maximum inhibition of phospholipase C was achieved

with 100μM BPB after 15min incubation. After 1min incubation, approximately 20% inhibition was observed. 50μM BPB would presumably cause even less inhibition. These workers used crude platelet extracts exposing phospholipase C, otherwise in the soluble fraction of intact platelets. The efficiency of BPB in inhibiting phospholipase C and diglyceride lipase would be expected to be reduced in intact platelets. On the other hand, phospholipase A_2, being in the membrane, may be more accessible to BPB. Experiments examining the effects of BPB on exogenous phospholipase C are presented and discussed in Chapter 6. Phenothiazines are not specific, and may also affect α-receptors or α-receptor linked events (Cocks et al., 1981) and thus other enzymes will be inhibited.

Bearing these cautionary remarks in mind, it is of interest that BPB or TPCK not only did not prevent TXB_2 formation, but rather, stimulated an even greater increase in thromboxane production when adrenaline potentiated the ADP response, or when high concentrations of ADP alone were used. According to other reports thromboxane production should be reduced in the presence of these concentrations of BPB since it affects cyclooxygenase and/or synthetase in addition to its direct action on phospholipase A_2. The assumption that phospholipase A_2 is the only source of arachidonic acid would lead one to predict a complete inhibition of thromboxane production by BPB.

There is, however, more than one source of arachidonic acid in the platelet. PI breakdown by phospholipase C and the action of a lipase on the resultant diglyceride can produce arachidonic acid (Bell et al., 1979). Phosphatidic acid could also act as a source of arachidonic acid but would also require a phospholipase A_2 for its release. It is possible that in the presence of BPB or TPCK, stimulation of phopholipase C and/or diglyceride lipase may still occur. This stimulation could be caused by potentiation of ADP by adrenaline, probably involving the α_2-receptor, or could be caused by high concentrations of ADP alone. With the blockade of other phospholipid changes, arachidonate release could actually be stimulated via the phospholipase C-diglyceride lipase pathway(Fig5.1).

Vargaftig et al. (1980b) noted that TPCK did not lower thromboxane production as expected and postulated an alternative pool of arachidonic acid. Vargaftig (1976; 1977) also reported potentiation of TXA_2 formation by TPCK and by BPB under certain conditions. BPB and TPCK have similar chemical properties in that both can alkylate a histidine residue, accounting for the direct inhibition of phospholipase A_2. The two inhibitors may also share non-specific effects. Nevertheless, it is interesting that they both have the same effect on adrenaline potentiation of ADP-induced aggregation.

This chapter contributes the further information that the addition of adrenaline to subthreshold ADP leads to potentiation of TXA_2 formation in the presence of TPCK or

BPB. Further, clonidine, a partial α_2-receptor agonist but not phenylephrine a partial α_1-receptor agonist, mimics this property of adrenaline. It could be argued that adrenaline might overcome the inhibition of phospholipase A_2 directly by perhaps increasing Ca^{2+} availability. However, as BPB is a direct enzyme inhibitor, binding to the active site, it would seem more likely that adrenaline stimulates an alternative mechanism for arachidonate release such as the phospholipase C/lipase pathway (Fig.5.1). The early formation of TXA_2 would support this theory since phospholipase C is thought to be more rapidly activated than phospholipase A_2 (Billah et al., 1980).

The phenothiazines, chlorpromazine and trifluoperazine exhibited characteristics which differed from BPB and TPCK with respect to adrenaline potentiation. The phenothiazines inhibited arachidonic acid release from all sources. At 50μM, chlorpromazine was expected to inhibit the second wave of aggregation stimulated by ADP or adrenaline and to inhibit thromboxane-dependent potentiation of ADP by adrenaline. Although this was indeed confirmed, thromboxane-independent potentiation was observed in the presence of these inhibitors. The mechanism of this thromboxane-independent aggregation would therefore seem to be independent of certain events subject to α-receptor interaction and calmodulin-dependent enzymes.

It is believed that the contribution of the diglyceride lipase pathway for arachidonate release is minor while

phospholipase A_2-activated release predominates in response to thrombin (Imai et al., 1982). While the predominant mechanism of arachidonate release caused by ADP is not known, it has been shown that ADP increases phosphatidic acid turnover (Lloyd et al., 1973a,b). In the presence of phenothiazines, it might be possible that adrenaline is able to potentiate diglyceride kinase activity, which would increase phosphatidic acid formation due to ADP, since there is no evidence of arachidonic acid release due to diglyceride lipase activity. This must await biochemical analysis to be confirmed. Alternately, effects of adrenaline on calcium influx or mobilization need to be considered.

Rao et al. (1980b) reported on the influence of trifluoperazine and BPB on platelet aggregation and disaggregation. They concluded that adrenaline could correct the inhibition of phospholipase A_2 by BPB. They were, however, studying re-aggregation of dissociated platelets which is a different situation to an adrenaline-potentiated ADP response as studied here.

Despite the very different results obtained with different types of phospholipase A_2 inhibitors, some general conclusions can be drawn. Firstly, adrenaline has effects on ADP-induced aggregation which lead to increased thromboxane production, possibly through increased arachidonic acid release. Secondly, adrenaline enhances platelet responses by a pathway independent of thromboxane production as indicated in the previous chapter. In the presence of phenothiazines the potentiation was obviously

independent of thromboxane production. Stimulation of arachidonic acid release from diacylglycerol may be possible in the presence of BPB or TPCK. Diversion of diacylglycerol to arachidonic acid may account for the decrease in the extent of the primary phase and the early onset of the second phase.

An association between adrenergic receptors and phospholipase C activation has been shown in other cells. Abdel-Latif(1974) for example, found that catecholamines caused a stimulation of ^{32}Pi incorporation into PA and PI of the iris muscle of the rabbit. The relationship between α_1-receptor activation and the PI effect has been discussed in Chapter 4. It is, however, the α_2-receptor which seems to be involved in the adrenaline-platelet interaction.

Adrenaline has been shown to cause changes in phospholipids in association with release (Deykin and Snyder,1973) and more specifically, adrenaline and ADP have been found to increase production of diacylglycerol, but only after 120sec (Kawahara et al.,1983b). Very recently, it has been found that adrenaline alone fails to cause breakdown of PI independently of release(Clare and Scrutton,1984) but the potentiating effects of adrenaline on ADP have yet to be analysed in this manner. Since ADP alone can stimulate the PI cycle, adrenaline may potentiate ADP by enhancing the effect of ADP on PI turnover or alternately, by enhancing events consequent upon PI metabolism caused by ADP. Finally, the combined effects of

adrenaline and ADP on calcium fluxes may be involved and need to be investigated.

CHAPTER 6

THE EFFECTS OF EXOGENOUS PHOSPHOLIPASE C ON PLATELETS.

6.0 SUMMARY

Aggregation and release due to exogenous phospholipase C were examined in the presence of several inhibitors used in the previous studies of adrenaline potentiation. Aggregation was found to be prostaglandin-independent whereas a second wave of release was found to be thromboxane-dependent. Aggregation by exogenous phospholipase C in the presence of BPB, however, was inhibited by indomethacin. Calcium and c'AMP appear to regulate the response to phospholipase C.

Phospholipid analysis of platelets treated with exogenous phospholipase C in the presence or absence of the inhibitors BPB or verapamil showed that PC could be broken down without an increase in PA production. It is possible that under these conditions, diglyceride lipase is more active, releasing arachidonic acid which accounts for increased TXB_2 production in the presence of BPB. Diacylglycerol could have a pivotal role in the release mechanism, and if unavailable for the protein phosphorylation reaction (Kawahara et al., 1980) release could be inhibited. The first phase of release caused by exogenous phospholipase C in resuspended platelets could be due to the formation of diacylglycerol from PC.

Spermine was found to cause inhibition of the action of exogenous phospholipase C itself, preventing diacylglycerol production and hence phosphatidic acid production. Trimetoquinol did not inhibit phosphatidic

acid production but caused inhibition of phospholipase C-induced aggregation.

The results using exogenous phospholipase C in the presence of BPB or a combination of BPB and verapamil are consistent with diglyceride lipase being more active in the presence of BPB. It remains to be shown that this is the pathway by which ADP, and adrenaline potentiated ADP, trigger responses in the presence of BPB.

6.1 INTRODUCTION

The activity of endogenous phospholipase C in platelets is significant because of its role in the conversion of inositol phospholipid to diacylglycerol in stimulated platelets (Lloyd et al., 1972; 1973a,b; 1974; Lapetina and Cautracasas, 1979; Rittenhouse-Simmons, 1979). Diacylglycerol may be metabolized by two alternative pathways and may also be involved in protein phosphorylation (Kawahara et al., 1980). Through the action of a kinase, diacylglycerol can be converted to phosphatidic acid. Alternatively, the action of a lipase can supply arachidonic acid from this same intermediary (Bell et al., 1979). The relative importance of these two enzymes in normal platelet function is under debate.

Exogenous phospholipase C also increases the amount of phosphatidic acid in the platelet membrane (Chap et al., 1979). The substrate is not inositol phospholipid as in thrombin-treated platelets, but is believed to be phosphatidylcholine (PC), the most predominant phospholipid after sphingomyelin. Platelet membrane phospholipids are

distributed asymmetrically. PS,PI and PE are present largely on the interior of the membrane of inactivated platelets. PC on the exterior of the membrane is an obvious site of attack. Although the phospholipid substrates for endogenous and exogenous phospholipase differ, investigation of the action of exogenous phospholipase C on platelets could still be worthwhile because of its ability to produce diacylglycerol and phosphatidic acid.

Chap et al. (1971) reported that phospholipase C derived from Clostridium welchii induces aggregation of human platelets with the production of ceramides and diaclyglycerols. Cell lysis follows slowly as indicated by lactate dehydrogenase release. Schick and Yu (1974) also studied the effects of this phospholipase C on platelets. They showed that PC and SPH were hydrolyzed, and platelet release of serotonin, ADP and platelet factor 4 occurred. These platelets were found to be intact by ultrastructural analysis after treatment with phospholipase C. It was also verified that the enzyme hydrolysis was due to the phospholipase and not due to any bacterial contaminant. PE and PS were degraded when more extensive hydrolysis occurred but PI was not found to be affected.

It is difficult to investigate endogenous phospholipase C activity since agonists known to stimulate phospholipase C also stimulate phospholipase A_2. Thrombin (Lapetina et al., 1981a) and collagen (Broekman et al., 1980) for example, stimulate both phospholipases. ADP has also been shown to stimulate phospholipase C activity

within 30sec of contact with platelets (Lloyd et al., 1972) with the activation of phospholipase A_2 occurring secondarily (Best et al., 1980). The calcium ionophore A23187 also stimulates both phospholipases but is an inefficient stimulus for diacylglycerol generation, and in fact requires the formation of cyclooxygenase products and ADP to stimulate phospholipase C (Rittenhouse,1984).

Another method of examining the role of phospholipase C activity involves the use of specific inhibitors. Unfortunately, no such specific inhibitor has been found to date. Walenga et al. (1980) demonstrated that several serine esterase inhibitors did, in fact, inhibit PI-specific phospholipase C, but also inhibited phospholipase A_2. Since the serine esterase inhibitors blocked arachidonic acid release by A23187, an enzymatic step beyond calcium mobilization is inhibited, which in the case of A23187 is likely to be phospholipase A_2 (Rittenhouse-Simmons, 1981). Phenothiazines may inhibit phospholipase C but they also inhibit phospholipase A_2(Rao et al., 1980b). Trimetoquinol has recently been reported to be a phospholipase C inhibitor (Huzoor-Akbar et al., 1982) as have some polyamines (Nahas and Graff, 1982) but the specificity of these compounds is yet to be determined.

The following studies were carried out to characterize the aggregation of platelets and associated release achieved with exogenous phospholipase C, and examine the effect of several inhibitors which have been previously used with adrenaline. The effects of inhibitors on

phospholipase C treated platelets and adrenaline potentiated responses of platelets are compared.

6.2 METHODS AND MATERIALS

6.2.1 Preparation of Platelets

Blood was obtained and plasma prepared as decribed in Chapter 2, with citrate as the anticoagulant for the preparation of platelet rich plasma (PRP) or acid-citrate-dextrose for the preparation of washed platelets and resuspended platelets. Some studies were performed with PRP and some with unwashed platelets suspended in a modified Tyrode's solution. This resuspension method was essentially the same as the washing procedure described except that the wash step consisted of rinsing the top layer of the platelet pellet, instead of resuspending the whole pellet in washing fluid, thus eliminating one centrifugation step. The modified Tyrode's solution had the following composition:NaCl 8.0g/l, KCl 0.2g/l, $NaHCO_3$ 1.0g/l, NaH_2PO_4 0.05g/l, $MgCl_2$ 0.009g/l, $CaCl_2$ 0.022g/l, albumin 3.5g/l, glucose 1g/l; pH 7.4, 37°C.

6.2.2 Platelet Studies

Aggregation, measurement of ATP release, TXB_2 radioimmunoassay and phospholipid analyses were undertaken according to the methods described in Chapter 2.

6.2.3 Materials

Phospholipase C, Type I was obtained from Sigma and had a specific activity of 8.1U/mg solid. Thrombin(Parke-

Davis, bovine origin) was also used as an aggregating agent.

The inhibitors used were aspirin, indomethacin, BW755C, verapamil (Isoptin), p-bromophenacyl bromide, phenoxybenzamine, dibenamine, spermine, chlorpromazine, promethazine and trifluoperazine. Their sources and usage were as described in Chapter 2.

6.3 RESULTS

6.3.1 Characteristics of the Response to Phospholipase C

Exogenous phospholipase C (0.1 mg/ml) caused aggregation in PRP after a variable delay of up to 5min (Fig. 6.1). Resuspended platelets were more sensitive to phospholipase C with a threshold of 5-10µg/ml, and the aggregation response was faster (Fig. 6.1). Release of nucleotides in PRP commenced slowly and reached a peak after aggregation was almost complete (Fig. 6.1). In contrast to PRP, the release reaction in resuspended platelets occurred in two phases. The first phase was very rapid, coinciding with the shape change, and the second phase appeared about one minute after, or later if the overall reaction was slow for that particular donor. The release reaction was more extensive in resuspended platelets than in PRP (Fig 6.1). Aggregation of platelets due to phospholipase C in either medium was associated with the formation of small amounts of TXB_2. For example, in PRP, phospholipase C (0.1mg/ml) caused the formation of 0.2-2.0 pmol $TXB_2/10^7$ platelets.

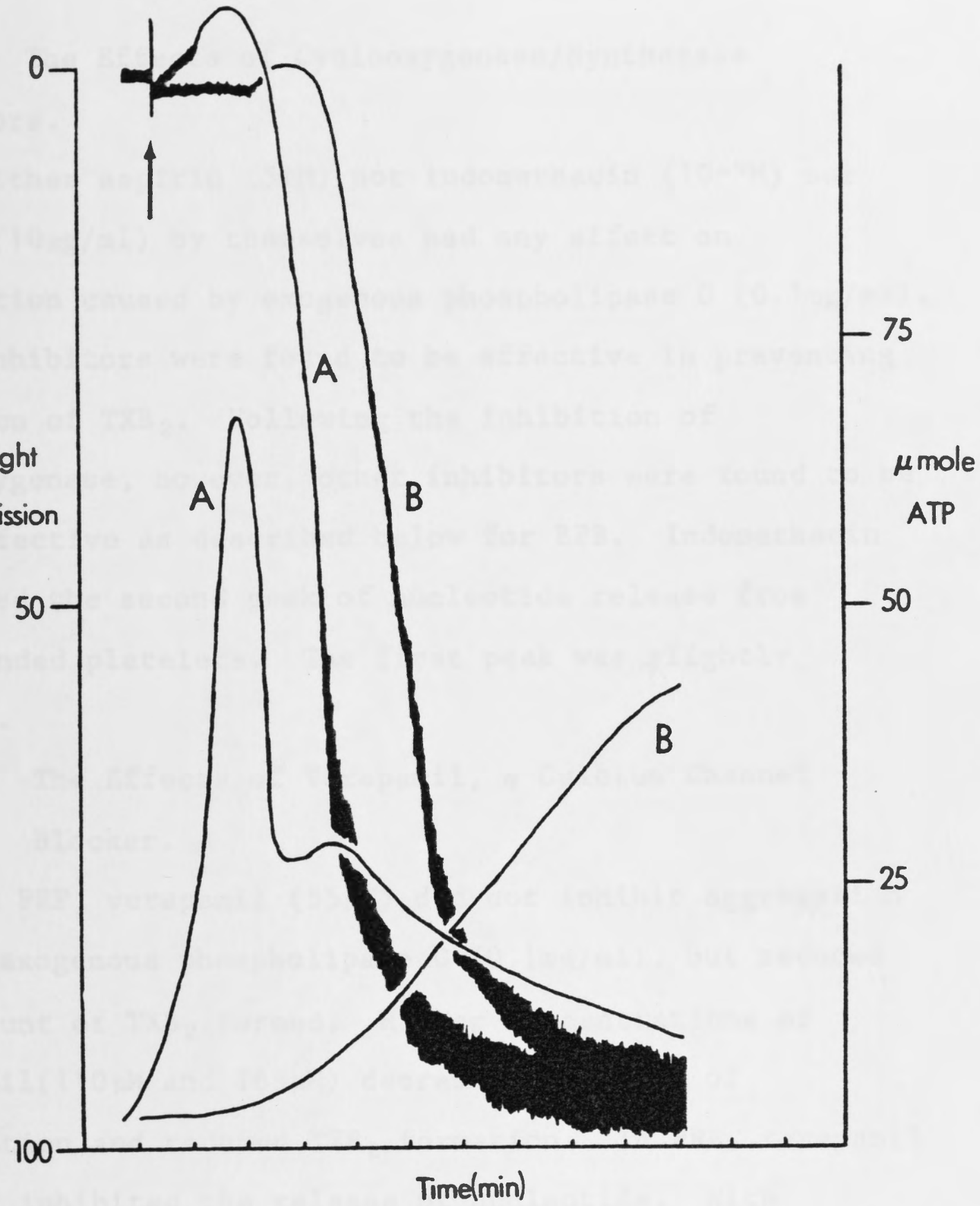

Figure 6.1 Platelet aggregation and release caused by exogenous phospholipase C.

Platelets were suspended in platelet rich plasma(B) or in modified Tyrode's solution (see Methods)(A). Aggregation(upper traces) was acheived with 0.1mg/ml (0.8U/ml)(B) and 0.01mg/ml(0.08U/ml)(A) of phospholipase C Release of nucleotide was assessed in comparison to ATP standards using a lumiaggregometer(lower traces). TXB_2 levels obtained on completion of aggregation were A,0.6pmol/10^7 platelets and B,1.0pmol/10^7 platelets.

6.3.1.1 The Effects of Cyclooxygenase/Synthetase Inhibitors.

Neither aspirin (5mM) nor indomethacin (10^{-4}M) nor BW755C (10µg/ml) by themselves had any effect on aggregation caused by exogenous phospholipase C (0.1mg/ml). These inhibitors were found to be effective in preventing formation of TXB_2. Following the inhibition of cyclooxygenase, however, other inhibitors were found to be more effective as described below for BPB. Indomethacin inhibited the second peak of nucleotide release from resuspended platelets. The first peak was slightly reduced.

6.3.1.2 The Effects of Verapamil, a Calcium Channel Blocker.

In PRP, verapamil (55µM) did not inhibit aggregation due to exogenous phospholipase C (0.1mg/ml), but reduced the amount of TXB_2 formed. Higher concentrations of verapamil(110µM and 165µM) decreased the rate of aggregation and reduced TXB_2 formation. In PRP, verapamil (165µM) inhibited the release of nucleotide. With resuspended platelets, the first phase of release was markedly reduced, while the second was increased (Fig 6.2). Nifedipine (10µM) had a similar effect to verapamil (165µM) (results not shown).

6.3.1.3 The Effects of Bromophenacyl Bromide (BPB)

In both resuspended platelets and PRP, BPB (100µM) partially inhibited aggregation caused by exogenous phospholipase C while increasing TXB_2 formation (Fig 6.3).

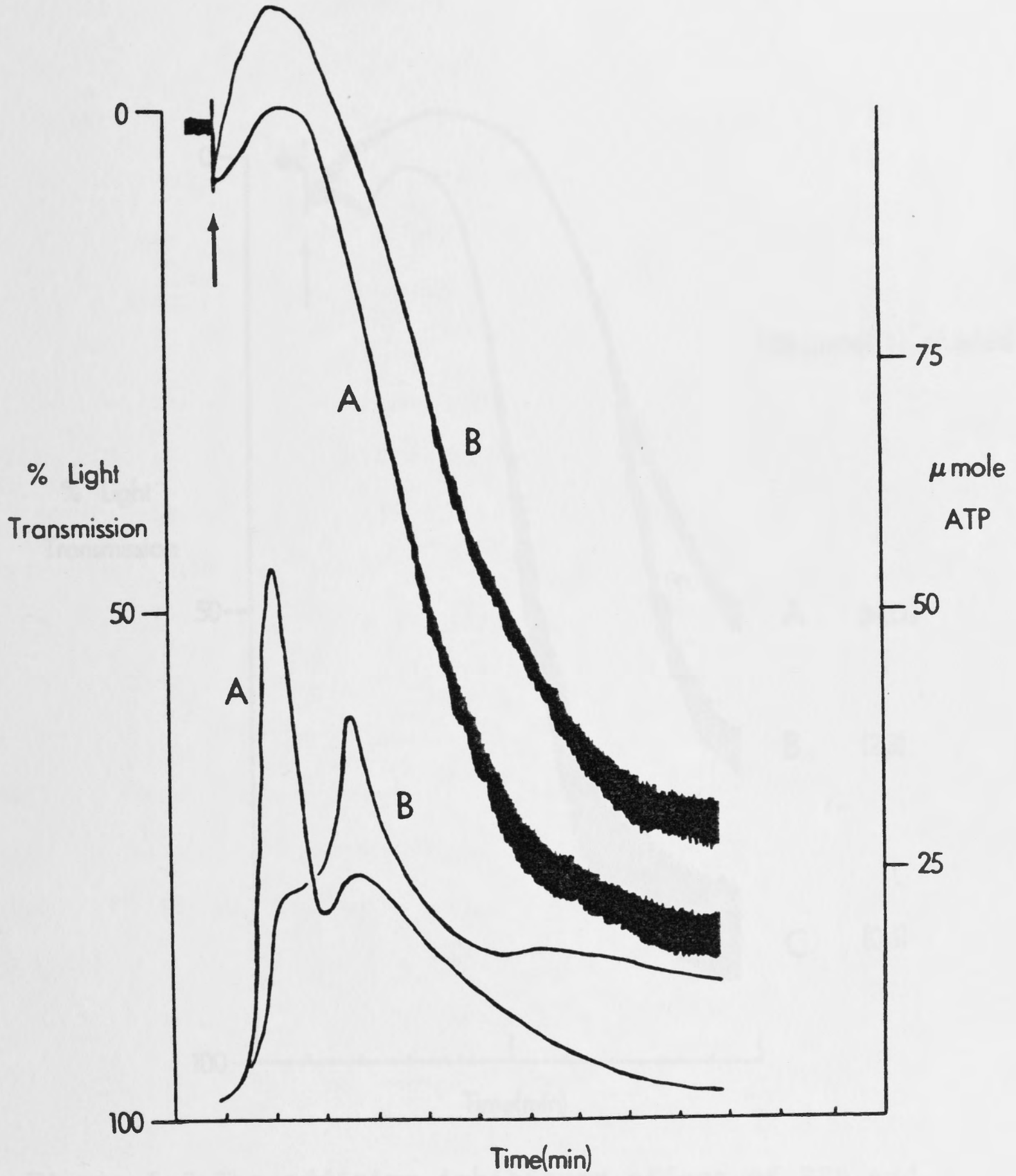

Figure 6.2 The effects of verapamil on aggregation and release due to exogenous phospholipase C.

Platelets were suspended in modified Tyrode's solution and were aggregated with 0.05mg/ml phospholipase C without inhibitor(A) or with 75μg/ml verapamil(B)(upper traces). Release of nucleotide was assessed in comparison to standard ATP using a lumiaggregometer(lower traces). TXB_2 levels were A,0.5pmol/10^7 platelets and B,0.3pmol/10^7 platelets.

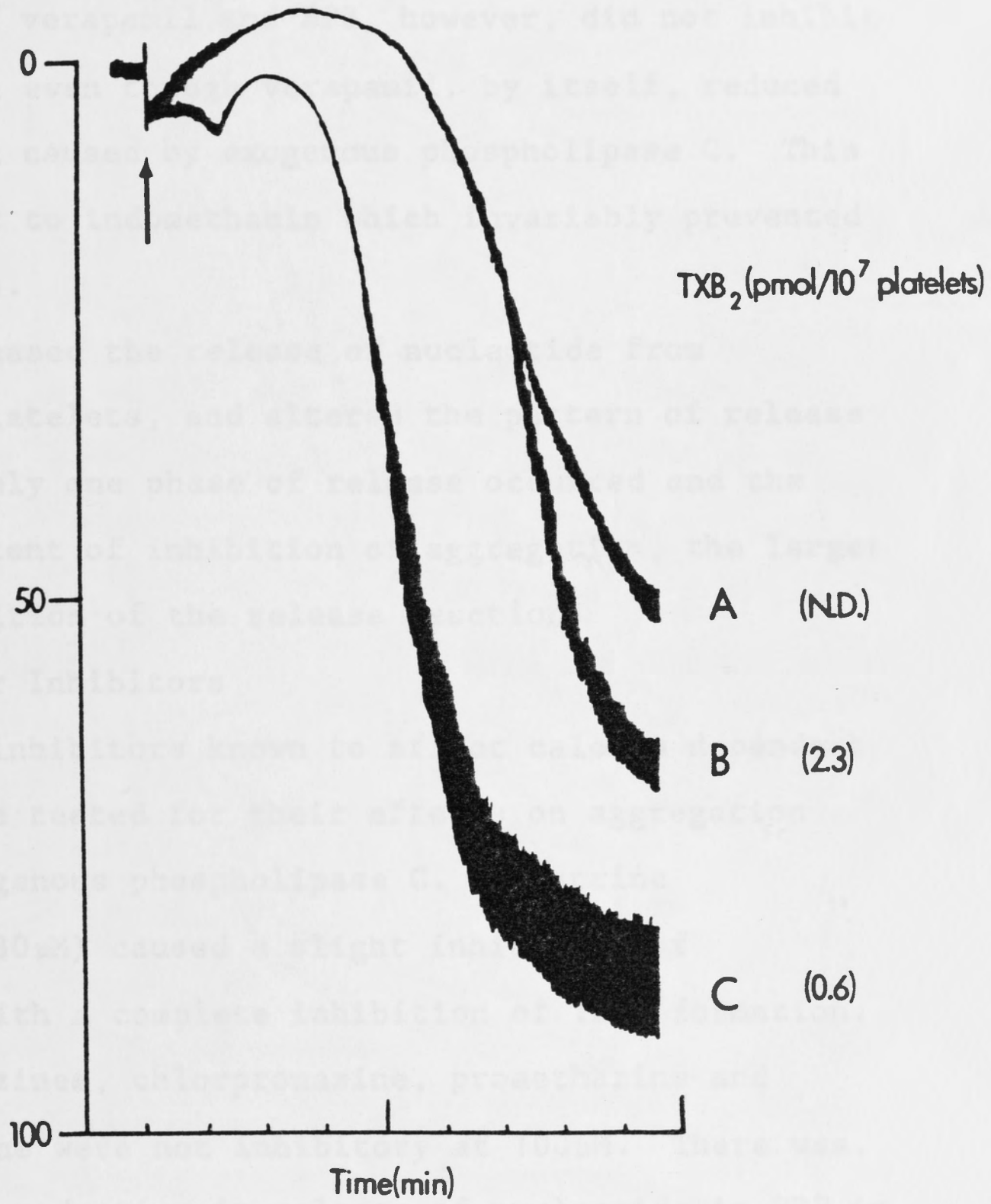

Figure 6.3 The additive inhibitory effect of BPB and indomethacin on aggregation due to phospholipase C.

Platelet rich plasma (400,000 platelets/µl)prepared from citrated blood was incubated for 1min with (A) 10^{-4}M indomethacin + 100µM BPB, (B) 100µM BPB and (C) DMSO. Phospholipase C(0.1mg/ml) was added at the arrow. ND=not detected.

Preincubation with a combination of indomethacin and BPB completely inhibited TXB_2 formation(Fig.6.3). The combination of verapamil and BPB, however, did not inhibit TXB_2 formation even though verapamil, by itself, reduced TXB_2 formation caused by exogenous phospholipase C. This is in contrast to indomethacin which invariably prevented TXB_2 formation.

BPB decreased the release of nucleotide from resuspended platelets, and altered the pattern of release (Fig 6.4). Only one phase of release occurred and the larger the extent of inhibition of aggregation, the larger was the inhibition of the release reaction.

6.3.1.4 Other Inhibitors

Several inhibitors known to affect calcium dependent reactions were tested for their effects on aggregation caused by exogenous phospholipase C. Mepacrine (quinacrine)(30μM) caused a slight inhibition of aggregation with a complete inhibition of TXB_2 formation. The phenothiazines, chlorpromazine, promethazine and trifluoperazine were not inhibitory at 100μM. There was, however, some reduction in release of nucleotide in PRP in the presence of phenothiazines. Phenoxybenzamine(40μM) and dibenamine (40μM) also reduced release but not aggregation. In resuspended platelets, this reduced release of nucleotide occurred in one peak only, well after the shape change, as aggregation was proceeding. Prostacyclin (10μg/ml) and theophylline(1mM) partly inhibited aggregation if the concentration of phospholipase C was so

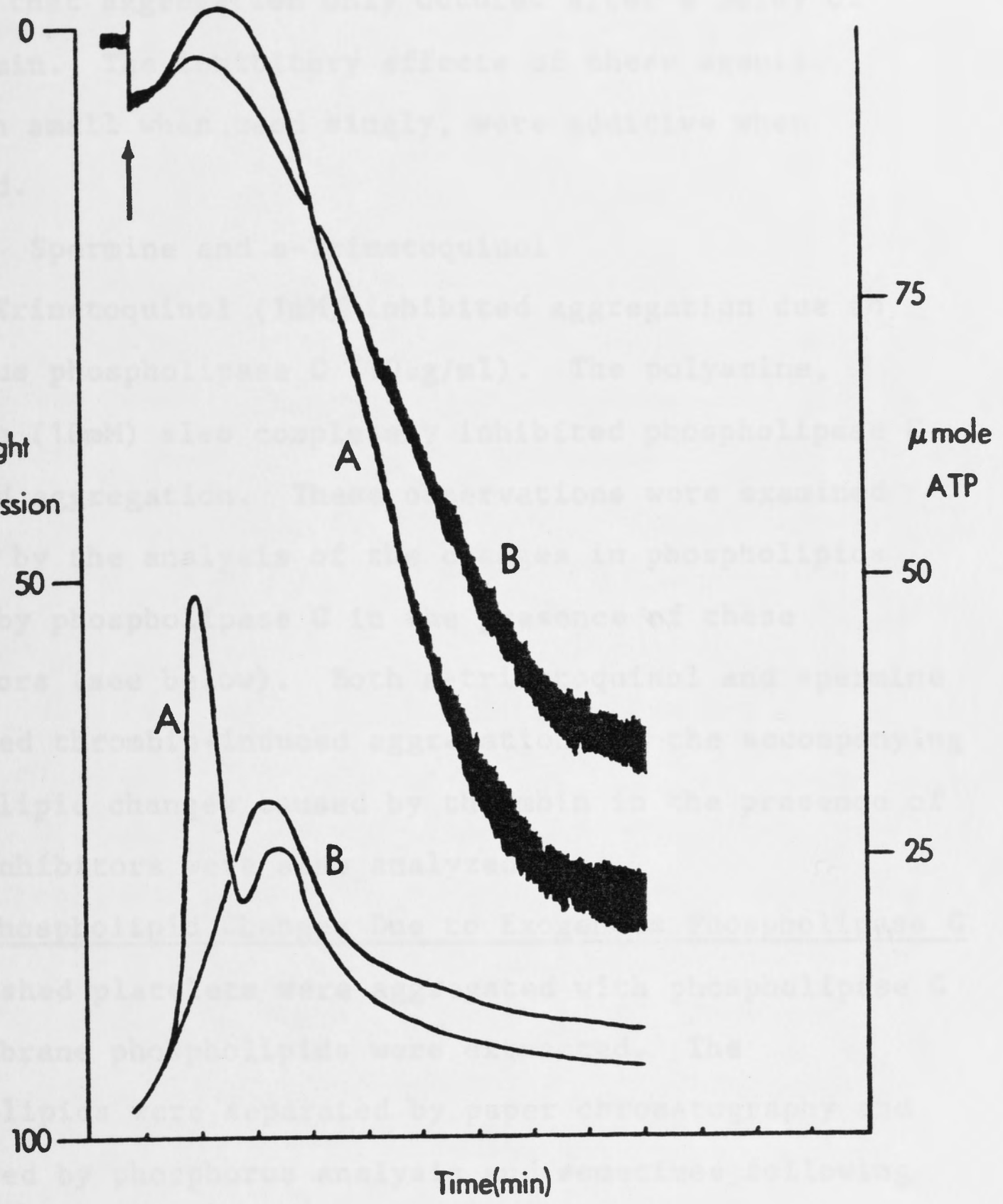

Figure 6.4 The effects of bromophenacyl bromide on aggregation and release due to exogenous phospholipase C.

Platelets were suspended in modified Tyrode's solution and were aggregated with 0.05mg/ml phospholipase C without inhibitor(A) and with 50µM BPB(B)(upper traces). Release of nucleotide was assessed in comparison to standard ATP using a lumiaggregometer(lower traces). TXB_2 levels were 0.5pmol/10^7 platelets for both traces.

reduced that aggregation only occured after a delay of about 4min. The inhibitory effects of these agents, although small when used singly, were additive when combined.

6.3.1.5 Spermine and s-Trimetoquinol

s-Trimetoquinol (1mM) inhibited aggregation due to exogenous phospholipase C (10µg/ml). The polyamine, spermine (10mM) also completely inhibited phospholipase C mediated aggregation. These observations were examined further by the analysis of the changes in phospholipids caused by phospholipase C in the presence of these inhibitors (see below). Both s-trimetoquinol and spermine inhibited thrombin-induced aggregation and the accompanying phospholipid changes caused by thrombin in the presence of these inhibitors were also analyzed.

6.3.2 Phospholipid Changes Due to Exogenous Phospholipase C

Washed platelets were aggregated with phospholipase C and membrane phospholipids were extracted. The phospholipids were separated by paper chromatography and estimated by phosphorus analysis and sometimes following ^{3}H-arachidonic acid incorporation. The phospholipids analyzed were PC, SPH, PI, PE, PS and PA. The distribution of these lipids on a typical chromatogram is given in Fig 6.5. The lysolipids, lysophosphatidylcholine (LPC) and lysophosphatidylethanolamine (LPE) were tentatively identified in addition to these.

The major change caused by phospholipase C was an increase in PA. A typical phospholipid analysis is given

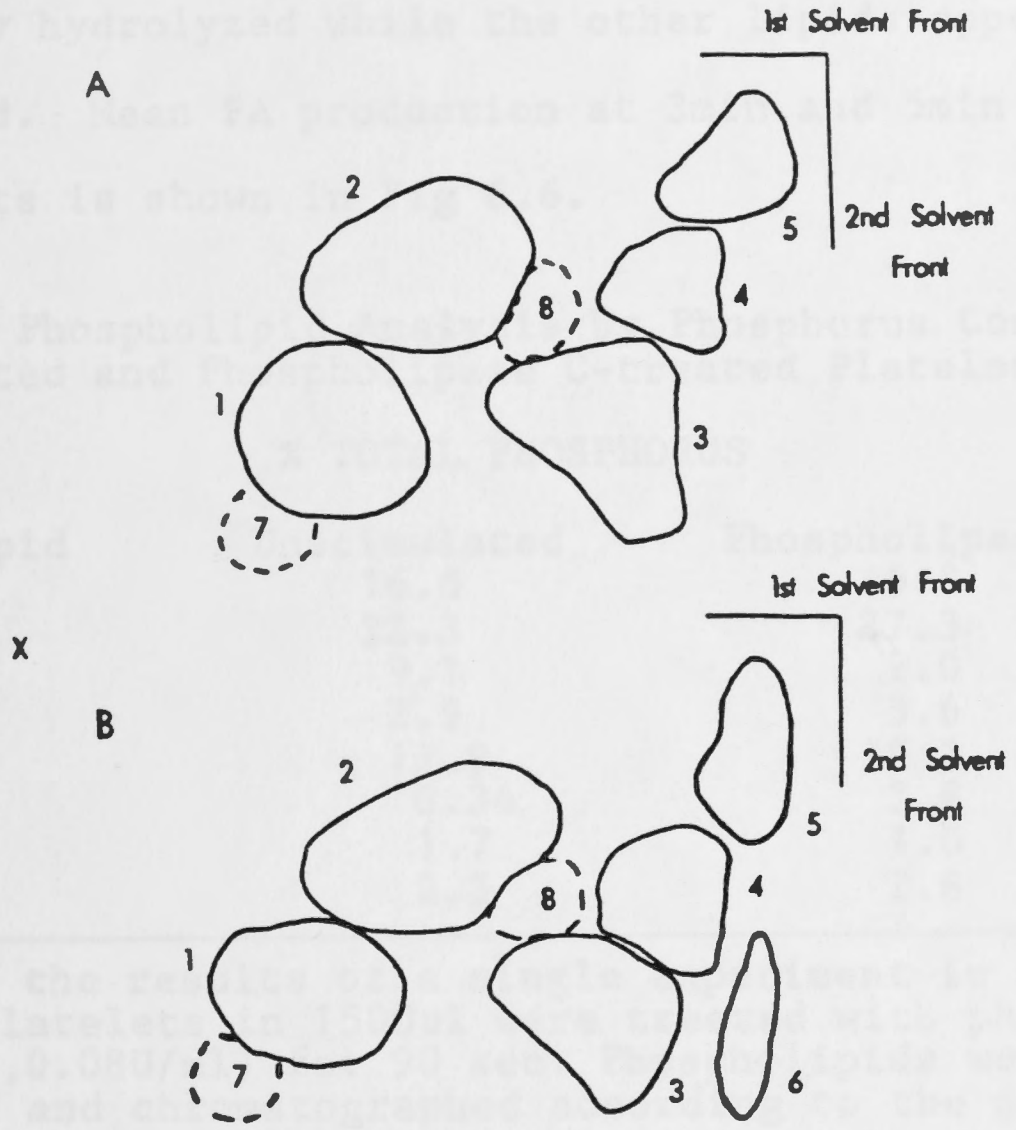

Figure 6.5 Chromatograms of unstimulated(A) and phospholipase C-activated(B) platelets.

Chromatograms were run on Whatman SG-81 paper, first in chloroform:methanol:5N ammonia(64:34:4) and then in chloroform:diisobuylketone:pyridine:methanol:acetic acid:formic acid:water(23:20:25:20:8:1:3). The phospholipids are identified(see Ch.7) as:1.sphingomyelin SPH 2.phosphatidylcholine PC 3.phosphatidylserine PS 4.phosphatidylinositol PI 5.phosphatidylethanolamine PE 6.phosphatidic acid PA 7.lysoPC 8.lysoPE.

in Table 6.1. PA increased 10-fold in 3min compared with the area (unstained) in a corresponding control chromatogram of unstimulated platelets. PC is the lipid measurably hydrolyzed while the other lipids appear unaffected. Mean PA production at 3min and 5min for three experiments is shown in Fig 6.6.

Table 6.1 Phospholipid Analysis by Phosphorus Content for Unstimulated and Phospholipase C-treated Platelets.

	% TOTAL PHOSPHORUS	
Phospholipid	Unstimulated	Phospholipase C
SPH	16.5	16.3
PC	32.3	27.3
PS	9.1	9.0
PI	2.9	3.6
PE	17.9	19.3
PA	0.34	3.8
LPC	1.7	1.0
LPE	2.3	2.8

These are the results of a single experiment in which 5.7×10^9 platelets in 1500µl were treated with phospholipase C(10µg/ml,0.08U/ml) for 90 sec. Phospholipids were extracted and chromatographed according to the methods described.

6.3.3 The Effects of Inhibitors on Phospholipid Changes due to Exogenous Phospholipase C

The effects of BPB and verapamil on changes in PC and PA changes caused by phospholipase C are shown in Table 6.2. Only PC and PA are shown as there were no detectable changes in the other phospholipids. The data includes estimation of phospholipids by ^{3}H-arachidonic acid incorporation (2 experiments) and by phosphorus assay (4 experiments). Changes caused by phospholipase C in the

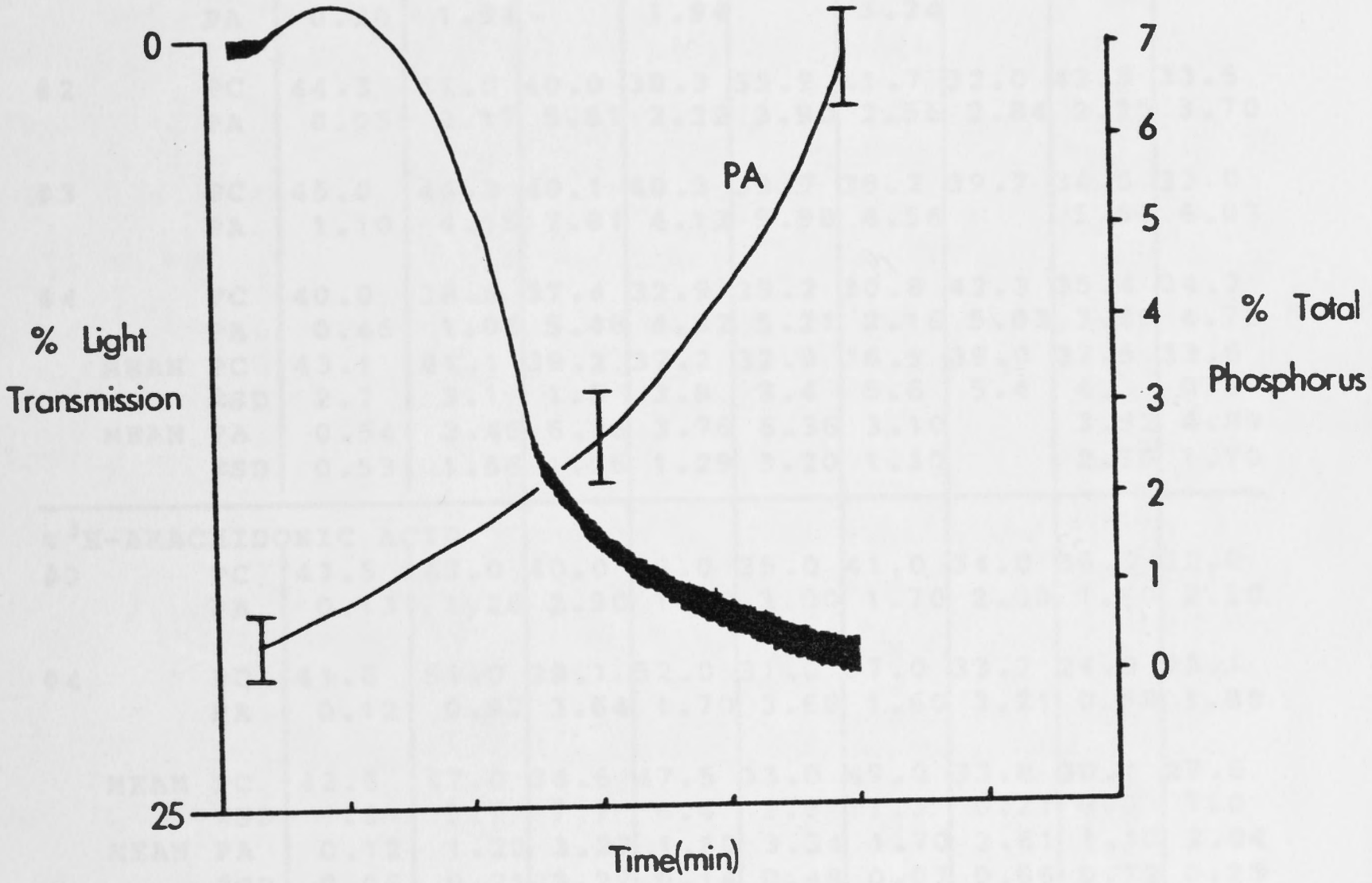

Figure 6.6 Production of phosphatidic acid during platelet aggregation due to phospholipase C.

Platelets were washed (methodB) and resuspended at 2-3x10^9 platelets/ml. PA was estimated by phosphorus assay of the spot separated on two-dimensional chromatography and shown in terms of %total phosphorus as the mean of three experiments ±SD depicted as bars.

presence of BPB (50μM) or verapamil (50μg/ml) or a combination of these two inhibitors are shown.

Table 6.2 Changes in PC and PA due to Exogenous Phospholipase C(PLC) in the Presence of BPB and/or verapamil.

% TOTAL PHOSPHORUS

		Unstim.	PLC		PLC +BPB		PLC +verap.		PLC +BPB/verap	
Exp.	PL	C	3	5	3	5	3	5	3	5min
#1	PC	47.4	36.5		27.6		36.8			
	PA	0.28	1.94		1.94		3.24			
#2	PC	44.3	41.0	40.0	38.3	35.9	41.7	32.0	42.5	33.5
	PA	0.05	2.17	5.81	2.32	3.90	2.56	2.84	2.35	3.70
#3	PC	45.0	44.2	40.1	40.3	33.7	38.2	39.7	34.6	33.0
	PA	1.10	4.15	7.81	4.13	9.98	4.56		5.60	6.83
#4	PC	40.0	38.0	37.4	32.9	29.2	30.8	42.3	35.4	34.2
	PA	0.46	1.06	5.48	4.82	5.21	2.18	5.03	3.80	4.73
MEAN	PC	43.1	41.1	39.2	37.2	32.9	36.9	38.0	37.5	33.6
	±SD	2.7	3.1	1.5	3.8	3.4	5.6	5.4	4.3	0.6
MEAN	PA	0.54	2.46	6.36	3.76	6.36	3.10		3.92	4.89
	±SD	0.53	1.56	1.26	1.29	3.20	1.30		2.30	1.70
* ^{3}H-ARACHIDONIC ACID										
#3	PC	43.5	43.0	40.0	43.0	35.0	41.0	34.0	36.0	32.0
	PA	0.13	1.26	2.90	1.50	3.00	1.70	2.00	1.60	2.20
#4	PC	41.6	51.0	29.1	52.0	31.0	57.0	33.7	24.2	23.1
	PA	0.12	0.97	3.64	1.70	3.68	1.60	3.21	0.58	1.88
MEAN	PC	42.6	47.0	34.6	47.5	33.0	49.0	33.8	30.1	27.6
	±SD	1.3	5.7	7.7	6.4	2.8	11.3	0.21	8.3	7.0
MEAN	PA	0.12	1.20	3.27	1.60	3.34	1.70	2.61	1.10	2.04
	±SD	0.06	0.21	3.27	0.14	0.48	0.07	0.86	0.72	0.23

In each experiment, 2-3x10^{9}platelets/ml were treated with phospholipase C(10μg/ml) for 3 or 5min. The last two experiments were analyzed for ^{3}H-arachidonic acid incorporation in addition to phosphorus analysis. Exp.#1 is excluded from the mean±SD because of limited data. BPB(50μM) and verapamil(50μg/ml) were added 1min before phospholipase C.

Using phosphorus estimation, it was found that exogenous phospholipase C achieved a greater hydrolysis of PC in the presence of BPB than in the absence of this

inhibitor. This was found in each experiment and is reflected in the mean. The large standard deviations are due to wide differences in responsiveness of different platelet preparations used in each experiment. PA production does not appear to account for this reduction in PC. Breakdown of PC by exogenous phospholipase C was also increased in the presence of verapamil, but not by as much as in the presence of BPB. PA production was not always increased in the presence of verapamil or BPB. In the presence of a combination of BPB and verapamil, a similar pattern emerged as in the presence of each inhibitor alone, namely, increased breakdown of PC but no further increase of PA production.

With ^{3}H-arachidonic acid incorporation, however, it was found that, in the presence of the combination of BPB and verapamil there was a greater loss of arachidonic acid from PC which did not appear in PA, compared to the effects of exogenous phospholipase C in the presence of each inhibitor singly. The changes in ^{3}H-arachidonic acid incorporation due to exogenous phospholipase C in the presence of these inhibitors, in general, revealed a different pattern to the results obtained using phosphorus estimation. No change in ^{3}H-arachidonic acid incorporation in PC was evident after 3min and there was only a small decrease in radioactivity by 5min. Changes in the presence of inhibitors were, therefore more difficult to detect.

Phospholipid analysis of phospholipase C-treated platelets in the presence of trimetoquinol or spermine is

shown in Table 6.3. Spermine, and to a lesser extent, trimetoquinol, inhibited the breakdown of PC by exogenous phospholipase C. The two inhibitors differed in their effect on PA production in that spermine (10mM) inhibited PA production while trimetoquinol (2mM) did not.

Table 6.3 Changes in PC and PA due to Exogenous Phospholipase C in the presence of Trimetoquinol or Spermine.

PL	% TOTAL PHOSPHORUS Unstimulated	Phospholipase C (PLC)	PLC + trimetoquinol (2mM)	PLC + spermine (10mM)
PC	31.4	18.2	24.6	34.4
PA	0.60	4.9	5.3	0.40

The results shown are from one experiment, and were confirmed in another. 3.4×10^9 platelets were treated with phospholipase C(100µg/ml) for 8min in the presence or absence of each inhibitor, preincubated for 1min.

6.3.4 The Effects of Inhibitors on Phospholipid Changes due to Thrombin

Washed platelets (method A) were aggregated with thrombin in the presence of the same inhibitors previously examined for their effects on phospholipase C-induced phospholipid changes. Two experiments were performed, one with 0.14U thrombin/10^8 platelets and another with 0.08U thrombin/10^8 platelets. Phospholipids were quantitated by ^{3}H-arachidonic acid incorporation and by phosphorus assay. BPB partially inhibited aggregation and verapamil almost totally inhibited aggregation by thrombin. Total

inhibition of aggregation by thrombin was achieved with the combination of BPB and verapamil. In Table 6.4 it can be seen that PA production due to thrombin was not prevented by BPB nor by verapamil. Also PI breakdown due to thrombin was not greatly affected by these inhibitors. Since only two experiments were performed, this data cannot be analyzed further.

Table 6.4 Changes in PC,PI and PA due to Thrombin in the Presence of BPB and/or Verapamil.

%^{3}H-ARACHIDONIC ACID

PL	Unstimulated	Thrombin		Thrombin +BPB		Thrombin +verap.		Thrombin +BPB/verap.
		3	5	3	5	3	5	3
PC	55.9	62.0	50.3	55.8	58.1	59.0	51.0	58.4
PI	8.73	5.6	3.7	6.8	5.9	5.4	4.9	6.8
PA	0.21	3.6	4.2	2.2	4.2	3.8	4.3	2.6

%TOTAL PHOSPHORUS

PC	43.0	44.8	38.9	45.5	39.7	38.8	38.6	42.8
PI	5.0	3.0	3.5	3.4	2.6	3.4	4.4	3.1
PA	0.47	1.9	2.5	2.7	2.0	3.9	2.9	1.8

$3.5x10^9$platelets/ml were treated with thrombin(5U/750µl) and incubated for 3 or 5min, in the presence or absence of BPB(50µM) or verapamil(50µg/ml) or a combination of the two.

Phospholipid analysis was also performed on thrombin-activated platelets (0.4 U/10^8 platelets) in the presence of trimetoquinol or spermine. The results are shown in Table 6.5. With phosphorus analysis, 100mM spermine prevented PI breakdown and PA formation, whereas 10mM trimetoquinol did not inhibit PI breakdown nor PA

formation, even though both caused inhibition of aggregation.

Table 6.5 Changes in PI and PA due to Thrombin in the Presence of Trimetoquinol or Spermine.

PL	% TOTAL PHOSPHORUS			
	Unstimulated	Thrombin	Thrombin + trimetoquinol (10mM)	Thrombin + spermine (100mM)
PI	6.8	5.3	5.2	6.3
PA	0.38	1.9	2.5	0.56

2.4×10^9 platelets/ml were treated with thrombin(8U/750µl) and incubated for 3min in the presence of trimetoquinol(10mM) or spermine (100mM).

6.4 DISCUSSION

There have been several reports on the effects of phospholipase C (derived from Clostridium welchii) on human platelets in vitro (Schick and Yu, 1974; Chap et al., 1977; Kawahara et al., 1980; Huzoor-Akbar et al., 1982). In agreement with these reports, the present data shows that phospholipase C causes aggregation and release of nucleotides from platelets. Aggregation was independent of prostaglandin synthesis but was partially inhibited by verapamil, mepacrine, phenothiazines and agents which increase c'AMP. It is suggested, therefore, that calcium and c'AMP may regulate the platelet response to phospholipase C.

Phospholipase C caused aggregation and release in both PRP and resuspended platelets although higher concentrations of the enzyme were required in PRP, and the

pattern of release in PRP differed from that in modified Tyrode's solution. The reduced sensitivity of PRP may be attributable to the presence of other lipids and to protein in the plasma. The first and more rapid phase of release of nucleotide from resuspended platelets corresponds in time with 5-hydroxytryptamine release by phospholipase C reported by Schick and Yu (1974). This first phase of nucleotide release was inhibited by verapamil (165μM) or by BPB (50μM). It could be speculated that, in the presence of BPB, diacylglycerol is diverted, possibly by the lipase (Bell et al., 1979) such that it is unable to be linked to protein phosphorylation for the release reaction (Kawahara et al., 1980). This is supported by the data showing increased breakdown of PC in the presence of BPB, without conversion to PA.

If diacylglycerol is involved in the action of exogenous phospholipase C it would have to enter the platelet after the action of phospholipase C on externally located PC. Phospholipase C does not enter the cell but instead utilizes the externally located substrate as indicated by the experiments with ^{3}H-arachidonic acid. Phospholipase C caused very little change in ^{3}H-arachidonic acid in PC, while phosphorus analysis indicated significant hydrolysis of PC. These observations indicate that the substrate for phospholipase C is PC located in the outer membrane leaflet. Inner membrane PC contains a higher proportion of arachidonic acid while PC in the outer

membrane leaflet contains oleate and linoleate (Rittenhouse-Simmons and Deykin, 1981).

Both calcium mobilization and diacylglycerol production seem to be necessary for the full physiological response of the platelet(Kaibuchi et al.,1983). Verapamil may inhibit the release reaction by acting as a calcium antagonist which inhibits calcium-dependent reactions including protein phosphorylation.

The second phase of nucleotide release from resuspended platelets is thromboxane-dependent since it was inhibited by indomethacin. Aggregation is not thromboxane-dependent. Nevertheless, small amounts produced may contribute towards aggregation associated with the second phase of release. Thromboxane seems to contribute more to aggregation occurring in the presence of BPB since this was more sensitive to inhibition by indomethacin. Nevertheless, increases in TXB_2 observed in the presence of BPB or BPB and verapamil combined, were not able to overcome the inhibition of aggregation. A step beyond the formation of thromboxane therefore seems to be inhibited. If diacylglycerol lipase is indeed more active than the kinase in the presence of BPB, with resultant release of arachidonic acid, then the present observations can be explained.

The effects of BPB and verapamil on phospholipid changes caused by thrombin differ from their effects on phospholipid changes caused by exogenous phospholipase C. Thrombin activates endogenous phospholipase C in platelets,

forming diacylglycerol from phosphatidylinositol-4,5-bisphosphate (Broekman, 1984). Whereas exogenous phospholipase C was more active in the presence of BPB than in its absence, the activity of endogenous phospholipase C following thrombin activation was unaffected by BPB. The two phospholipases in question act on different substrates: PC in the case of the exogenous enzyme and PI in the case of thrombin activated phospholipase C. Thus there may be important differences in diacylglycerol formation and metabolism when platelets are activated by thrombin or exogenous phospholipase C, the latter enzyme requiring entry of diacylglycerol into the platelet.

Most of the agents examined for their ability to inhibit exogenous phospholipase C were only partially effective. Spermine(10mM) and trimetoquinol(1mM) were the only agents found to be completely inhibitory for exogenous phospholipase C. These two inhibitors differed in their effects on phospholipids. Spermine, but not trimetoquinol inhibited PA formation due to exogenous phospholipase C. Perhaps trimetoquinol inhibits a step in the activation pathway beyond diacylglycerol and PA formation. An interesting possibility is that trimetoquinol inhibits protein kinase C, since it does not inhibit diacylglycerol formation. Inhibition by spermine can be attributed to inhibition of diacylglycerol formation which is important for aggregation and release by phospholipase C.

The results of this chapter suggest that BPB could indeed, be affecting the phospholipase C pathway, perhaps

enabling diacylglycerol lipase to be more active. It would be interesting to examine the effects of BPB on the adrenaline potentiated response to ADP by platelets in terms of such phospholipid changes. If adrenaline can influence this pathway, it may well be of considerable physiological and pathological significance.

Chapter 7

THE EFFECT OF ADRENALINE ON PLATELET MEMBRANE PHOSPHOLIPIDS.

7.0 SUMMARY

The mechanism of prostaglandin-independent adrenaline potentiation may involve phospholipid changes, or alternately, could be associated with increased calcium uptake due to adrenaline. Since PI metabolism has been found to alter as a result of stimulation by other agonists, it was thought that adrenaline effects on PI metabolism should be examined.

Two solvent systems for a paper chromatographic technique for the separation and quantitation of PA and PI were compared. The second system was found to give a well resolved, compact spot compared to the more diffuse spot of the first system. This method was used for some preliminary experiments, examining adrenaline potentiation of ADP and thrombin.

The results are preliminary and therefore inconclusive but focus on the PI cycle and mobility within the different polyphosphoinositides as areas worthy of further investigation.

7.1 INTRODUCTION

Adrenaline potentiation of other platelet agonists *in vitro* may reflect the proposed important biological role of adrenaline in the preparation of platelets to react efficiently where required in wound repair. Besides an effect on arachidonic acid metabolism, the present work indicates that adrenaline potentiation can be independent of prostaglandin synthesis. Adrenaline, by its binding to

α_2-receptors (Grant and Scrutton,1979) possibly causes some kind of membrane change involving calcium mobilization(Rao et al., 1981a; Brass and Shattil, 1982). This calcium movement could be associated with phospholipid changes (Michell, 1975) or could be independent of phospholipids. Alterations to phospholipids have been observed during aggregation of human, horse and rabbit platelets induced by thrombin, collagen and ADP(Lloyd et al., 1973b; 1974; Broekman et al., 1980; Lapetina et al., 1981a).

Adrenaline has been found to increase PI synthesis in platelets, but only following completion of the release reaction (Deykin and Snyder,1973) and not in the presence of aspirin (Clare and Scrutton,1984). It is, however, the immediate post-receptor events in adrenaline activation which are of interest in the potentiation mechanism since potentiation can be acheived after only fifteen seconds or less prior contact with adrenaline. Both phospholipid turnover and changes in phospholipid proportions need to be assessed. Since PI is capable of cycling (Lapetina et al., 1981b) resynthesis is possible so that while PI can participate in a platelet reaction, its overall level may not alter, as was found for ADP (Lloyd et al.,1972). The evidence reported so far in this thesis indicates that adrenaline may affect phospholipase C-mediated PI metabolism when the ADP response is potentiated and this chapter focusses on the effects of adrenaline on this

aspect. These investigations, however, are only of a preliminary nature. Details of the development of the chromatographic procedure employed are also included.

A paper chromatographic method was chosen for the separation of phospholipids. With this technique, PI and PA could be separated and quantitated, but diacylglycerol could not. PI was considered to be important to assay for reasons which have been discussed previously. PA is a product of PI breakdown and its accumulation has been shown to be important in the response to thrombin (Lapetina et al., 1981). Increases in PA indicate that the enzyme diglyceride kinase is active as opposed to digyceride lipase. Silica gel-loaded paper has been reported to be suitable for separation of lipids from tissue extracts (Wuthier, 1966).

7.2 METHODS AND MATERIALS

Platelet preparation, aggregation, chromatography, ^{3}H-arachidonic acid incorporation and phosphorus assay were carried out according to the methods described in Chapter 2. The platelets were washed using method B with the inclusion of prostacyclin in each centrifugation. Aggregation was performed in a large cuvette in the aggregation module so that a sufficient number of platelets could be used to obtain measurable amounts of phospholipid. At least $1x10^{9}$ platelets(approx.30µg P) were required per incubate. For most experiments, 750µl platelet suspension containing approximately $3x10^{9}$ platelets was employed.

The lipid extraction procedure was modified from Bligh and Dyer(1959) to allow for the small volumes of each incubate. SG-81 paper from Whatman was used for two-dimensional chromatography to separate the phospholipids. The first pair of solvent mixtures employed (System A) used by Kramer et al.(1982), and modified from Wuthier(1966), was found to be unsuitable, especially for the recovery of measurable phosphatidic acid and was later substituted with a second pair of solvent mixtures (System B). A full comparison of chromatograms obtained with both pairs is reported in the Results section of this Chapter(7.3).

Phospholipids were quantitated by phosphorus assay (Rouser et al.,1966) and expressed as a percentage of total phosphorus extracted from the platelet incubate. Phospholipid turnover was assessed by determining the incorporation of ^{3}H-arachidonic acid.

The aggregating agents employed were adrenaline, ADP, and thrombin. The inhibitor used was BPB. Phosphatidic acid standards used included synthetic dioleoyl phosphatidic acid-Na salt(Sigma), dipalmitoyl phosphatidic acid-free acid(Sigma), lecithin-derived phosphatidic acid(Serdary) and phosphatidic acid(Supelco). Other standard phospholipids,PI(Supelco), PC, PE, PS and SPH(Sigma) were also used to identify chromatographic spots.

7.3 RESULTS

7.3.1 Chromatography with Solvent System A.

Two different solvent systems for two-dimensional chromatography on SG-81 paper were tested. The first system(A) separated platelet membrane lipids according to the pattern shown in Fig 7.1. PI resolved well in this system, but PA could not be observed, presumably because it constitutes such a small proportion of total phospholipids. The maximum lipid phosphorus load of this system is 10µg(Wuthier,1966) of which less than 0.1%(0.01µg) would be PA. This amount would not stain with rhodamine 6G, especially if the spot was diffuse. The localization of PA was therefore attempted with the use of commercial standards in quantities which would stain. Even so, the localization of platelet PA would not necessarily be coincident with PA derived from a source other than platelets(Wuthier,1976).

The mobilities of PA from three different sources in each phase of the solvent system are shown in Fig 7.2. All of these standards were unsatisfactory. The Serdary sample separated into three different spots in the second dimension. Dipalmitoyl-PA showed no movement at all in the second dimension and dioleoyl-PA moved high in both phases. PA moving rapidly in the second phase would overlap with cardiolipin and PE. Platelet PA could not be localized by this method.

Washed platelets($6x10^9$/ml) were treated with thrombin (0.2U/10^8platelets) for 2min, extracted and chromatographed in parallel with an extract from unstimulated platelets. The PI content of control and thrombin-stimulated platelets

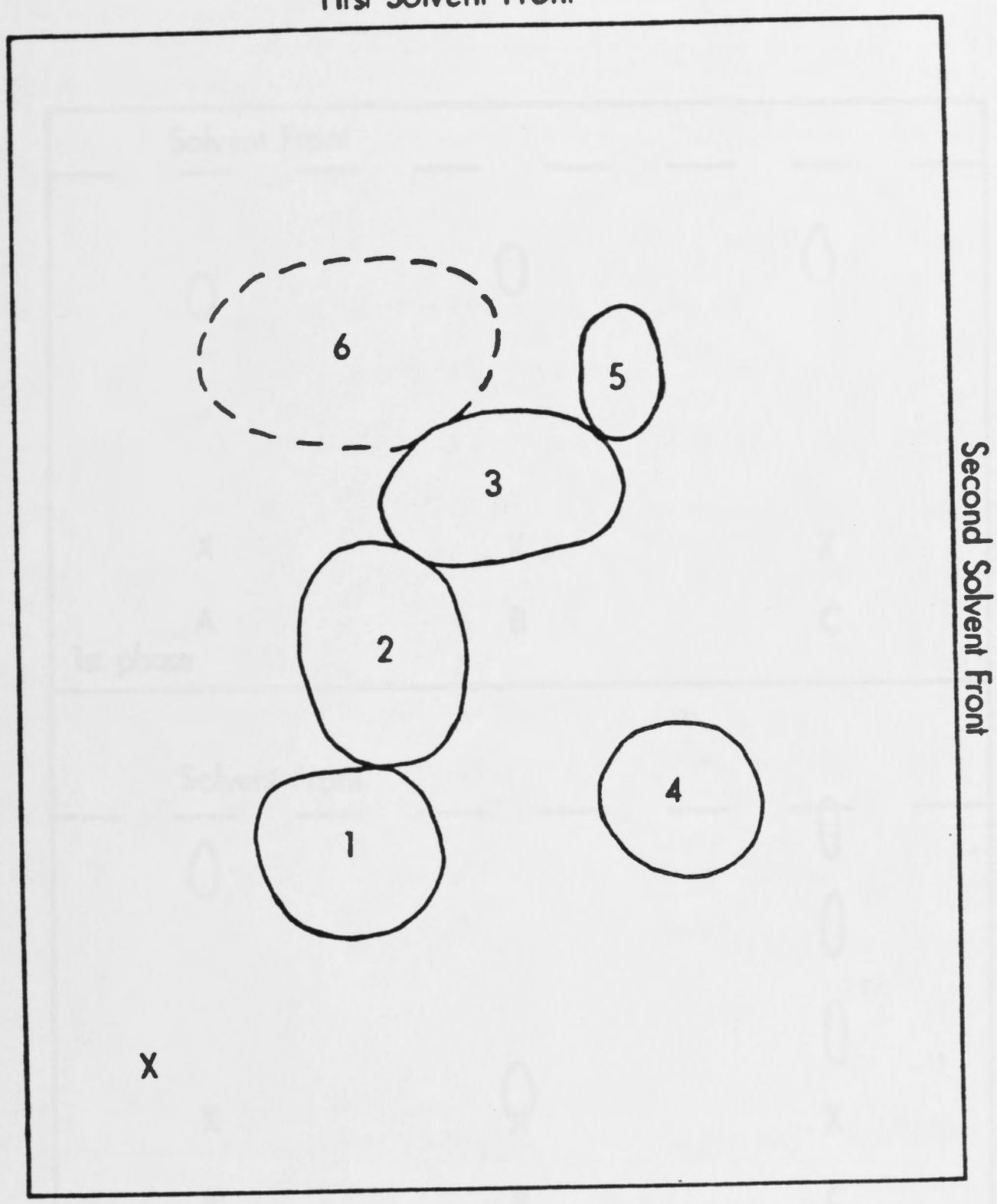

Figure 7.1 Chromatogram of platelet membrane lipids using solvent system A.

The lipids were extracted by a modification of Bligh and Dyer(1959). The chromatogram was run on Whatman SG-81 paper first in chloroform:diisobutylketone:methanol:acetic acid: water(45:30:15:20:4) for the long phase, and secondly in chloroform:diisobutylketone:pyridine:methanol:water(30:25: 35:25:8). The spots were stained with rhodamine 6G and identified as 1.SPH 2.PC 3.PS 4.PI 5.PE and 6.?PA by their colour under UV light and their comparison to standards. x=origin.

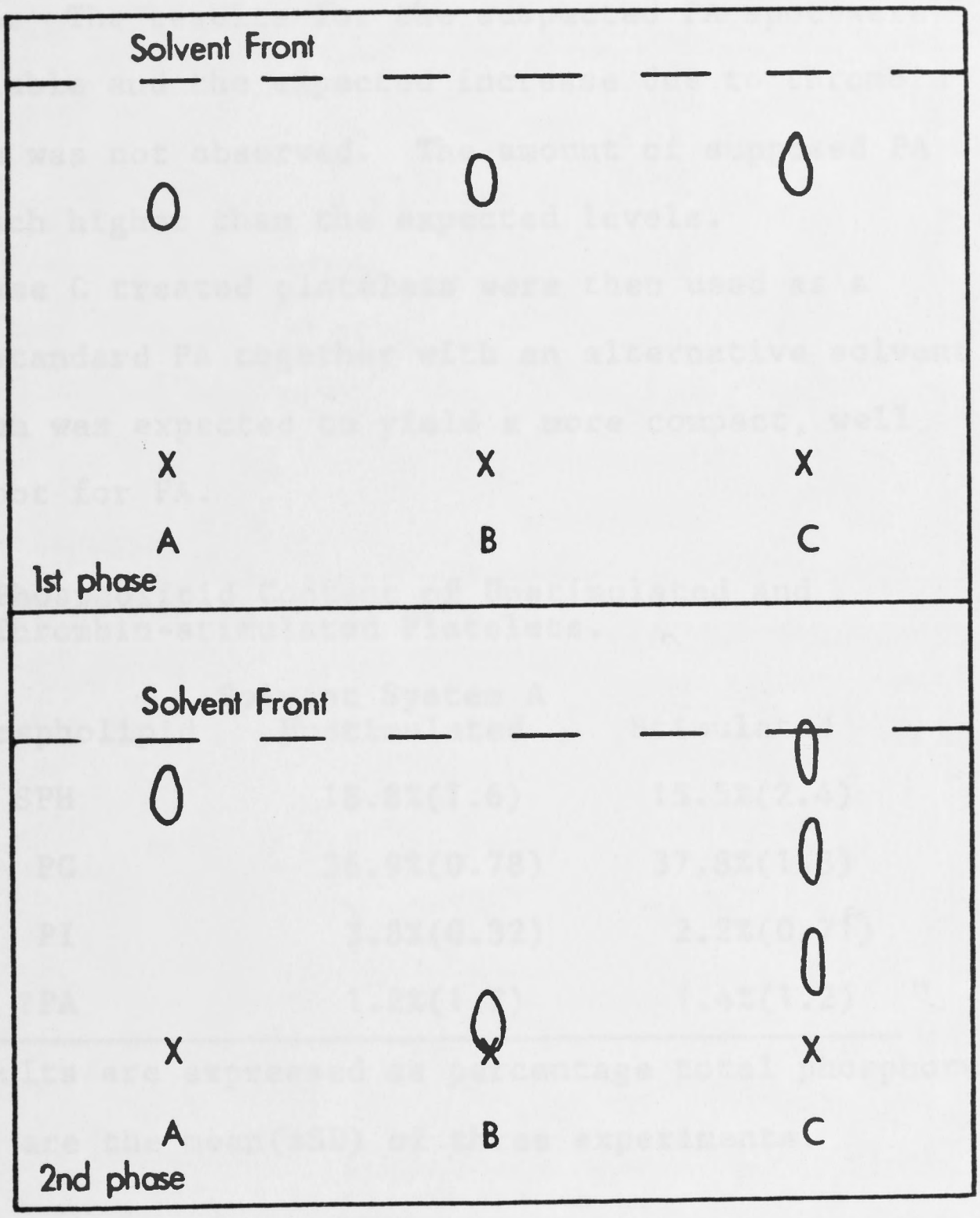

Figure 7.2 The mobilities of standard phosphatidic acids in each phase of solvent system A.

The PA samples were: A. synthetic dioleoyl PA-Na salt (Sigma) B. synthetic dipalmitoyl PA-free acid(Sigma) and C. egg lecithin derived PA(Serdary). The effect of the first phase solvents are shown in the upper chromatogram and the effect of the second phase solvents are shown in the lower chromatogram, run separately. The solvents of the two phases are as given for Fig 7.1.

is shown in Table 7.1 together with SPH and PC, and a possible PA. The results for the suspected PA spot were highly variable and the expected increase due to thrombin stimulation was not observed. The amount of supposed PA was also much higher than the expected levels. Phospholipase C treated platelets were then used as a source of standard PA together with an alternative solvent system which was expected to yield a more compact, well resolved spot for PA.

Table 7.1 Phospholipid Content of Unstimulated and Thrombin-stimulated Platelets.

	Solvent System A	
Phospholipid	Unstimulated	Stimulated
SPH	18.8%(1.6)	15.5%(2.4)
PC	36.9%(0.78)	37.8%(1.8)
PI	3.8%(0.32)	2.2%(0.71)
?PA	1.2%(1.7)	1.4%(1.2)

Results are expressed as percentage total phosphorus and are the mean(±SD) of three experiments.

7.3.2 Chromatography with Solvent System B.

The chromatograms of platelet membrane phospholipids using solvent system B are shown in Fig 7.3 and Fig 7.4. Chromatography of phospholipids of both thrombin-stimulated(Fig 7.3) and exogenous phospholipase C-stimulated platelets(Fig 7.4) revealed the appearance of a spot on the lower right hand corner not present in unstimulated platelets. This spot(numbered 6 in the Fig) is consistent with the position of PA in a chromatogram of rat tissue phospholipids illustrated by Wuthier(1976). A PA standard(Supelco) was subsequently found to

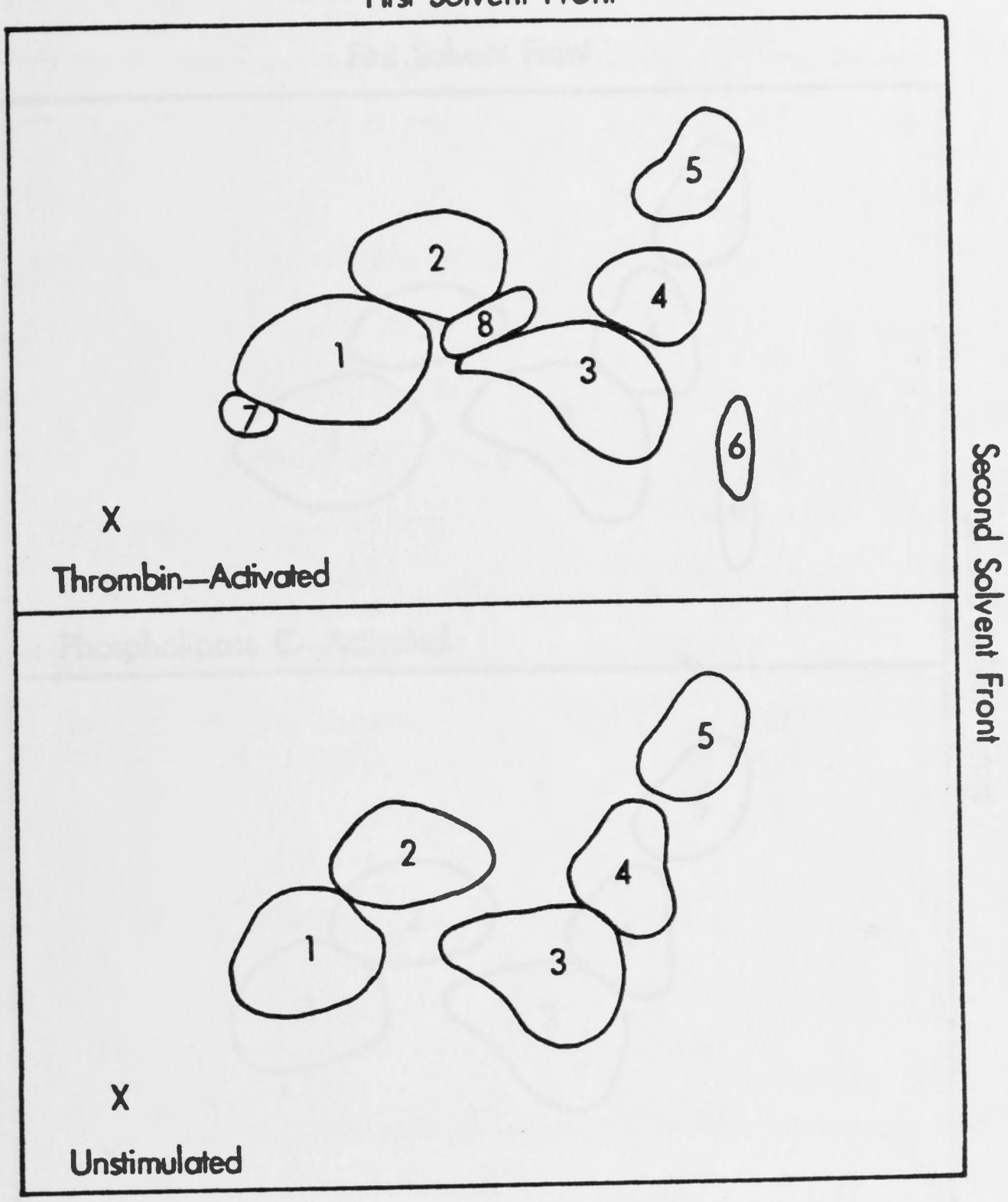

Figure 7.3 Chromatograms of platelet membrane lipids from unstimulated and thrombin-stimulated platlets.

Washed platelets ($3X10^9$/ml) were treated with thrombin (0.1U/10^8platelets) for 5min. The chromatograms were run on Whatman SG-81 paper, first in chloroform:methanol:5N NH_4OH (64:34:4) and secondly in chloroform:diisobutylketone: pyridine:methanol:acetic acid:formic acid:water(23:20:25: 20:8:1:3). The spots were stained with rhodamine 6G and identified as 1.SPH 2.PC 3.PS 4.PI 5.PE 6.PA 7.lysoPC 8.lysoPE. x=origin.

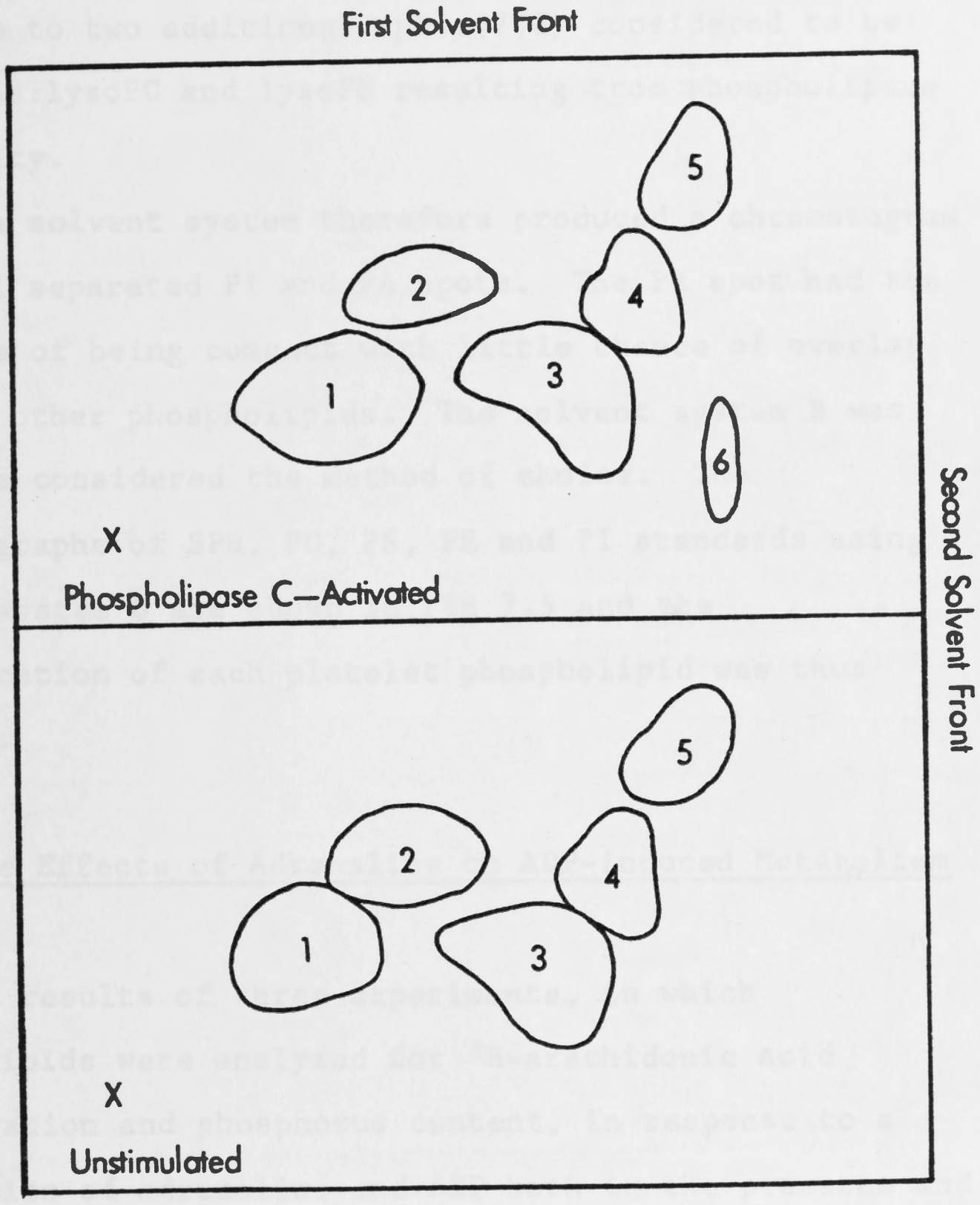

Figure 7.4 Chromatograms of platelet membrane lipids from unstimulated and phospholipase C-stimulated platelets.

Washed platelets($3X10^9$/ml) were treated with phospholipase C(10µg/ml) for 2min. The chromatograms were prepared as described for Fig 7.3. x=origin.

chromatograph in this position also. Thrombin treatment gave rise to two additional spots(7,8) considered to be lysolipids:lysoPC and lysoPE resulting from phospholipase A_2 activity.

This solvent system therefore produced a chromatogram with well separated PI and PA spots. The PA spot had the advantage of being compact with little chance of overlap with any other phospholipids. The solvent system B was therefore considered the method of choice. The chromatographs of SPH, PC, PS, PE and PI standards using solvent system B are shown in Fig 7.5 and the identification of each platelet phospholipid was thus verified.

7.3.3 The Effects of Adrenaline on ADP-induced Metabolism of PI.

The results of three experiments, in which phospholipids were analyzed for ^{3}H-arachidonic acid incorporation and phosphorus content, in response to a combination of adrenaline and ADP both in the presence and absence of BPB are shown in Table 7.2. These results are difficult to analyze for several reasons. First of all, the degree of incorporation of arachidonic acid in PA in unstimulated platelets is highly variable and stimulation by agonists causes variable effects. Stimulation may either decrease or increase this basal level of incorporation, or remain much the same, as in experiment #1.

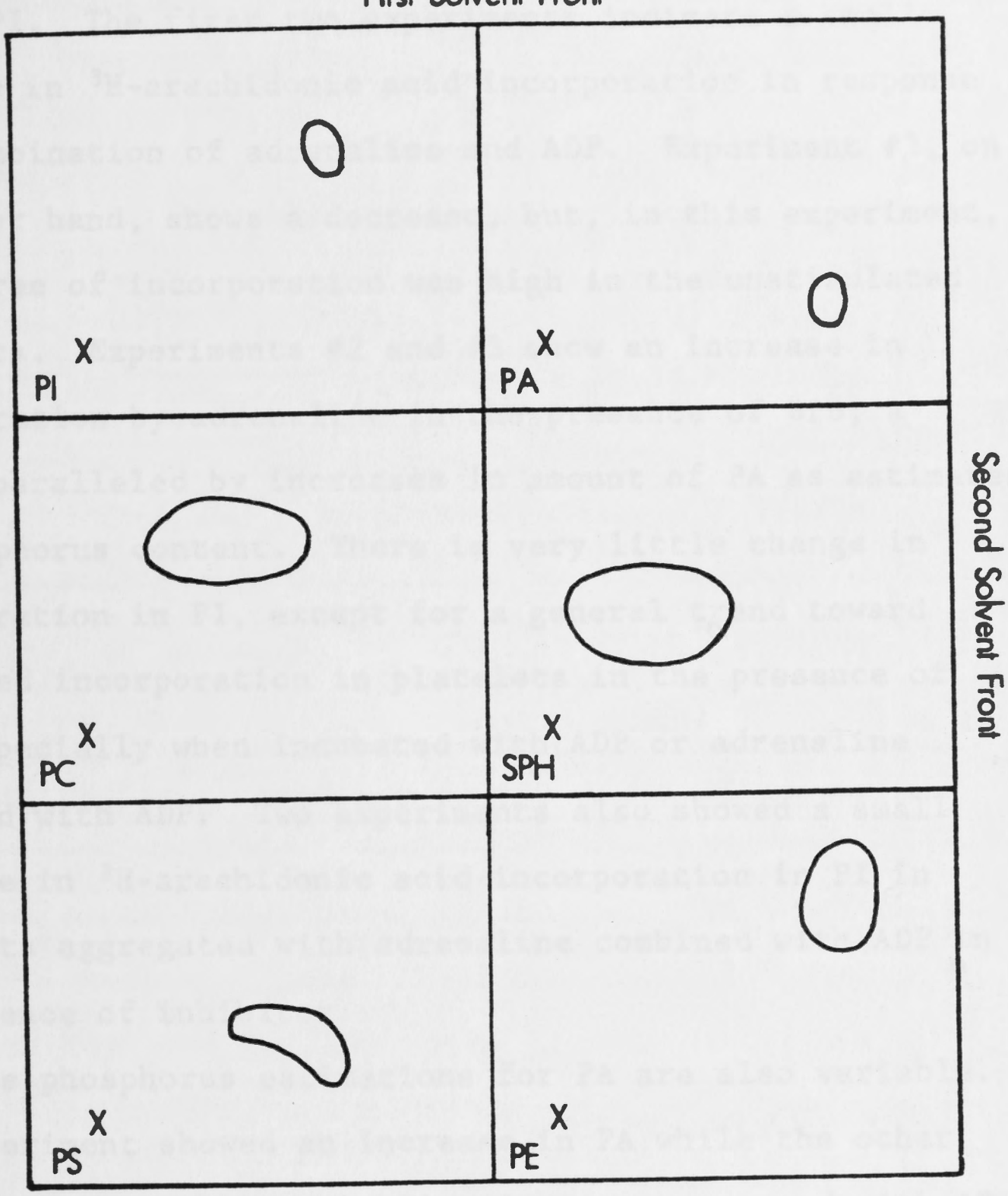

Figure 7.5 Chromatograms of standard phospholipids using solvent system B.

The standard phospholipids were chromatographed as described for Fig 7.3. The samples were:
PI(Supelco)5µg PA(Supelco)5µg PC,SPH,PS,PE,(Sigma)10µgea.
x=origin.

Secondly, there is no consistent pattern of changes in PA nor PI. The first two experiments indicate a small increase in ^{3}H-arachidonic acid incorporation in response to a combination of adrenaline and ADP. Experiment #3, on the other hand, shows a decrease, but, in this experiment, the degree of incorporation was high in the unstimulated platelets. Experiments #2 and #3 show an increase in incorporation by adrenaline in the presence of BPB, a result paralleled by increases in amount of PA as estimated by phosphorus content. There is very little change in incorporation in PI, except for a general trend toward increased incorporation in platelets in the presence of BPB, especially when incubated with ADP or adrenaline combined with ADP. Two experiments also showed a small increase in ^{3}H-arachidonic acid incorporation in PI in platelets aggregated with adrenaline combined with ADP in the absence of inhibitor.

The phosphorus estimations for PA are also variable. One experiment showed an increase in PA while the other showed no change, when the platelets were treated with ADP for 30 sec. The phosphorus estimations for PI are difficult to interpret.

Table 7.2 Phospholipid Changes During Adrenaline Potentiation of ADP-induced aggregation in the Presence or Absence of BPB.

%^{3}H-ARACHIDONIC ACID

Exp.No.	PL	C	No inhibitor Adr. 15s	Adr. 45s	ADP 30s	Adr/ADP 15/30s	BPB(50μM) Adr. 15s	Adr. 45s	ADP 30s	Adr/ADP 15/30s
#1	PA	0.23	0.27	0.32	0.25	0.40	0.19	0.21	0.32	0.38
	PI	6.64	6.57	6.48	6.82	6.32	6.93	7.14	7.82	9.30
#2	PA	0.69	0.57	-	1.40	1.55	2.13	-	0.40	1.55
	PI	6.03	5.98	-	5.71	7.11	6.30	-	6.08	7.13
#3	PA	2.34	0.99	0.45	0.90	0.49	2.86	0.59	1.45	0.41
	PI	6.38	6.80	7.21	5.98	7.38	7.12	6.17	8.34	7.01
%Phosphorus										
#2	PA	1.82	0.14	-	5.10	0.65	2.03	-	0.38	1.40
	PI	3.47	1.47	-	3.30	2.68	2.18	-	3.80	7.20
#3	PA	0.10	0.11	0.09	0.07	0.10	0.60	0.10	0.08	0.09
	PI	2.32	1.88	1.82	2.96	2.69	2.18	2.37	2.54	2.89

%^{3}H-arachidonic acid incorporation is shown for three experiments and %phosphorus for the last two experiments. 1-2x10^{9}platelets/ml were treated with adrenaline(Adr.) and/or ADP as indicated at the following concentrations:
#1 adrenaline 10^{-5}M, ADP 10^{-5}M.
#2 adrenaline $5X10^{-6}$M, ADP 10^{-5}M.
#3 adrenaline 10^{-4}M, ADP 10^{-4}M.
Fibrinogen(0.3mg/ml) was added 1min prior to the agonists.
Each figure is the result of a single estimation.
PL=phospholipid, s=sec, C=unstimulated platelets.

7.4 DISCUSSION

The results of this chapter, while of a preliminary nature, indicate that it is unlikely that adrenaline influences the total amounts of PI or PA. Some aspects of the data are conflicting, indicating that some aspects of the method could be improved.

Separation of PA and PI on Whatman SG-81 paper, using solvent system B, was adequate, provided excessive amounts were not applied, but was subject to variations in behaviour between different batches and small changes in climatic conditions. Platelet preparations were obtained from platelet-rich plasma supplied by the Red Cross Blood Bank. Variability in donors and in handling may have been responsible for the widely different unstimulated levels of arachidonic acid incorporation in PA. Other stimuli associated with handling procedures could account for high incorporation of arachidonic acid observed in PA in apparently unstimulated platelets.

ADP was expected to induce increased PA turnover, without altering the amount of PA (Lloyd et al.,1972), whereas thrombin was expected to increase the amount of PA. In the experiment where there was increased incorporation of arachidonic acid due to exposure to ADP for 30sec, there was also an increase in the amount of PA. On the other hand, where a large incorporation was observed in unstimulated platelets, ADP reduced this incorporation, possibly due to increased turnover. Further experiments are obviously needed to compare a wider range of platelet preparations.

Equally confusing results were obtained in experiments where adrenaline and ADP were added to platelets. It should be noted, however, that the two experiments which did show a rise in arachidonic acid incorporation were performed with platelets in which there was a low initial

incorporation. In the third experiment, where ADP reduced incorporation, adrenaline and ADP in combination reduced this incorporation further. All experiments therefore, show that adrenaline affects phospholipid metabolism, even if only to a small extent. Further replication of all experiments is necessary.

Phospholipase C may be activated in at least two different ways. Thrombin and other agonists interact with their receptors to directly activate phospholipase C which degrades PI (Lapetina et al., 1978) while the ionophore A23187 stimulates phospholipase C via thromboxane and ADP (Rittenhouse, 1984). A23187 mobilizes calcium which activates phospholipase A_2 to release arachidonic acid for thromboxane formation. If adrenaline could be shown to affect calcium mobilization, this may lead to activation of phospholipase C following secretion. When adrenaline potentiates another agonist, the effects of adrenaline on calcium may be sufficient to act synergistically with whatever pathways are triggered by the second agonist.

Increased labelling of PI, which seems to occur in the presence of BPB may indicate an increase in activity of the PI cycle (Lapetina et al., 1981b). Previous work, reported in Chapters 5 and 6, has indicated that BPB may inhibit diglyceride kinase, providing conditions suitable for increased lipase activity if diaclyglycerol production is increased.

The PI spot assayed by this technique may provide

insufficient evidence to determine the effects of adrenaline. It has been shown that both thrombin (Billah and Lapetina, 1982) and ADP (Vickers et al., 1982) cause rapid changes in amounts of phosphatidylinositol-4,5-bisphosphate which may chromatograph differently from PI, while the different pools of inositides could participate in reactions necessary for platelet activation in different ways. ADP did not cause any increase in the amount of PA associated with the decrease in PI. It was suggested by these authors that decreases in phosphatidylinositol-4,5-bisphosphate result in the mobilization of membrane-bound calcium. An alternative method which could differentiate between the different inositide classes in stimulated platelets might be more revealing in analyses of the effects of adrenaline on platelets.

Recently, adrenaline alone has been shown to not cause breakdown of PI in aspirin-treated platelets (Clare and Scrutton, 1984). The results presented here are inconclusive. The difficulty in observing measurable changes in phospholipids due to adrenaline stimulation may indeed arise because the potentiating effects of adrenaline are not attributable to breakdown of PI. The ability of adrenaline to stimulate calcium redistribution has not been excluded by recent work and adrenaline may influence calcium mobilization independently of the PI-effect.

CHAPTER 8

GENERAL DISCUSSION

8.1 DISCUSSION

Platelets make a vital contribution to the normal haemostatic process. There is also a great deal of evidence that increased platelet reactivity is important in the development of atherosclerosis, thrombosis and coronary artery disease. Vascular intimal thickenings have been shown to contain thrombus material (Mustard and Packham, 1975). Platelets adhere to exposed connective tissue following injury to the endothelium and subsequently release granule contents (Stemerman and Ross, 1972; Baumgartner et al., 1967; White, 1972b). Platelet derived growth factor stored in α-granules has been found to be an absolute requirement for the proliferation of smooth muscle cells and to promote migration of endothelial cells *in vitro* (Ross *et al.*, 1974; Wall *et al.*, 1978). It has thus been proposed that platelets participate in atherosclerotic plaque formation according to the Response-to-Injury Hypothesis (Ross and Glomset, 1976).

Increases in the rate or degree of response of the platelet can be brought about by the catecholamines, adrenaline and noradrenaline. Catecholamines may prepare platelets to respond more readily to other agonists. Adrenaline levels rise during even mild stress (Dimsdale and Moss, 1980) and this in part prompted the study of adrenaline interaction with platelets, since stress may be a major risk factor for coronary artery disease. Catecholamines are also increased in other risk factor conditions including cigarette smoking and hypertension.

Adrenaline may increase platelet reactivity, and thus contribute to atherogenesis and its complications. The mechanisms by which adrenaline increases the responsiveness of platelets has been the subject of this thesis. It is known that adrenaline interacts with its own specific cellular receptor on the platelet and this appears to be an α_2-adrenoreceptor(Grant and Scrutton,1979). The immediate post-receptor events in the platelet have not, so far, been determined.

The differing choices of anticoagulant necessary for *in vitro* studies of platelet reactivity has lead to difficulties in interpreting the two phases of the platelet response to adrenaline. The response in heparin, and only to a small extent in citrate, has previously been reported to be of the 'primary' type (O'Brien *et al.*,1969). Primary aggregation is prostaglandin-independent, while the major response in citrate is prostaglandin-dependent (Best *et al.*, 1980). It has been suggested that this 'secondary' aggregation is an artifact of citrated plasma (Mustard *et al.*, 1975) but the findings reported here in Chapter 3 indicate that secondary aggregation in response to ADP can occur if the heparin concentration is lowered sufficiently. The calcium chelating effects of an anticoagulant such as citrate are therefore not a prerequisite for prostaglandin-dependent or second phase aggregation.

Another criticism of the use of citrate as anticoagulant arises from the belief that it is the reduction in available calcium which reduces the primary

phase of adrenaline-induced aggregation. This may be so, but the presence of citrate does not, however, prevent adrenaline potentiating the response to other agonists (Grant and Scrutton, 1980a). Furthermore, this ability to potentiate is not inhibitable by aspirin (Rao et al.,1980a) and is therefore achieved by a prostaglandin-independent mechanism, which is expected to involve calcium redistribution without decreases in intra-platelet c'AMP (Rao et al., 1980b; 1981a). Citrated plasma was used in their experiments and in the experiments reported here in Chapters 4 to 7. Owen and Le Breton (1981) used a chlortetracycline probe to observe a release of intraplatelet membrane-bound calcium after adrenaline stimulation which was partially inhibited by indomethacin. Further evidence that calcium redistribution is involved in the thromboxane-independent mechanism has been obtained by Brass and Shattil(1982). These authors found that both ADP and adrenaline increased the number of surface membrane affinity sites for calcium in aspirin treated platelets. These agonists also increased the rate of calcium exchange into the intracellular pool without increasing the net amount of calcium.

Several authors have studied adrenaline potentiation of other agonists since it was first demonstrated by Ardlie et al.(1966). Chapter 3 reports on the extension of these in vitro studies to three or four agonists in combination, instead of the paired combinations employed previously. Circulating blood obviously contains several possible

platelet agonists albeit at low concentrations. For the multiple agonist experiments, heparin was chosen as the anticoagulant to more closely reflect the *in vivo* situation. The study demonstrated that aggregation of platelets was possible *in vitro* using concentrations of catecholamines which could be achieved *in vivo*. The previously published lowest levels achieving aggregation *in vitro* were still higher than in pathological states (Grant and Scrutton, 1980a). The reduced sensitivity to multiple agonists in citrate compared to heparin suggests that calcium may be involved in the potentiation mechanism.

The mechanism of adrenaline potentiation was explored in Chapter 4. The findings are in agreement with those of Rao *et al.*(1980a) to the extent that adrenaline stimulates both prostaglandin dependent and independent pathways when potentiating ADP. The results differed in being able to show by radioimmunoassay, small increases in cyclooxygenase activity due to adrenaline despite the presence of the cyclooxygenase inhibitor, aspirin, but only when high concentrations of arachidonate were used. In the presence of thromboxane inhibitors, higher concentrations of adrenaline are required to achieve the same extent of aggregation as in the absence of these inhibitors. Therefore, the thromboxane-dependent pathway represents the more efficient mechanism of adrenaline potentiation.

It is suspected that both the prostaglandin-dependent mechanism and prostaglandin-independent mechanism of adrenaline potentiation occur via stimulation of the α_2-

receptor since the activity of adrenaline can be mimicked by clonidine, a partial α_2-receptor agonist. The experiments using phenoxybenzamine and dibenamine are difficult to interpret, but do not preclude post-receptor events involving calcium mobilization.

Arachidonic acid release by adrenaline may involve activation of phospholipase A_2. The effects of putative phospholipase A_2 inhibitors were therefore examined and the findings are reported in Chapter 5. The surprising result with the use of BPB or TPCK was that, rather than enabling a prostaglandin-independent pathway to be examined, these inhibitors revealed that an alternative source of arachidonic acid could be released for thomboxane production. Despite the awareness of the lack of specificity of phospholipase A_2 inhibitors, which has been well documented in the case of BPB, it is nevertheless suggested that adrenaline stimulates the release of arachidonic acid via diglyceride lipase. This pathway is the only known source not involving phospholipase A_2 enzymes (Bell et al., 1979). BPB has been shown to inhibit diglyceride lipase in platelet extracts but not in intact platelets (Hofmann et al., 1982). The potentiation of thromboxane production in the presence of BPB or TPCK is in agreement with the work of Vargaftig (1976).

Thromboxane-independent potentiation of ADP-induced aggregation by adrenaline was shown to be possible in the presence of phenothiazines, inhibitors of several enzymes, including calmodulin-dependent enzymes. Because it has been shown that ADP increases PA turnover(Lloyd et al., 1972) it is conceivable that adrenaline increases kinase activity and thus PA formation, in the presence of phenothiazines, rather than stimulating arachidonic acid release. The findings of Chapter 5 are therefore consistent with those of Chapter 4 in that adrenaline may influence PI metabolism. This remains to be confirmed by biochemical analysis. It is also possible that adrenaline in the presence of other agonists may promote calcium mobilization independently of PI metabolism.

Since there was some doubt about the possibility of diglyceride lipase being active in the presence of BPB (Hofmann et al., 1982) the effects of exogenous phospholipase C in the presence of BPB were examined. Aggregation, release of nucleotide and phospholipid metabolism due to exogenous phospholipase C are the subject of Chapter 6. This Chapter also includes an investigation of possible inhibitors of phospholipase C and the effects of these on thrombin-induced aggregation and phospholipid metabolism.

Exogenous phospholipase C caused the formation of a small amount of TXA_2 associated with aggregation. In the

presence of BPB, TXA_2 formation increased while aggregation was partially inhibited. This parallels the increase in TXA_2 observed when adrenaline potentiates ADP. In the presence of BPB, release of nucleotide due to exogenous phospholipase C was largely inhibited but a small indomethacin-sensitive peak of release still occurred. Phospholipid analysis indicated increased external PC breakdown without a corresponding conversion to PA in the presence of BPB. In addition, phospholipase C-induced aggregation in the presence of BPB was inhibited by indomethacin. These data are consistent with diglyceride lipase being more active in the presence of BPB. This would increase arachidonic acid release from diacylglcerol and thus increase TXA_2 formation. It could also divert diacylglycerol away from PA production, providing a mechanism for the inhibition of threshold phospholipase C-induced aggregation by BPB. Interference at this level could alter phosphorylation reactions essential for release and aggregation.

Similar experiments were performed with thrombin replacing exogenous phospholipase C. Thrombin activates endogenous phospholipase C, forming diacylglycerol from phosphatidylinositol-4,5-bisphosphate (Broekman, 1984) Since it was found that BPB did not affect PI breakdown due to thrombin, and TXA_2 was not estimated, these experiments

neither confirmed nor denied that the lipase could be more active in the presence of BPB.

Inhibitors of phospholipase C-induced aggregation may have different mechanisms of action. For example, spermine prevented phosphatidic acid production but trimetoquinol did not. Perhaps trimetoquinol inhibits a step in the activation pathway beyond diacylglycerol and PA formation.

The above evidence, based on aggregation studies with associated TXA_2 formation and nucleotide release required support from biochemical analyses of membrane phospholipids which were subsequently undertaken. The important question of adrenaline effects on phospholipid metabolism, and in particular, PI turnover, in the presence or absence of certain inhibitors could only be answered by quantitation of the relevant phospholipids. Chapter 7 reports on the development of a paper chromatographic method for the separation of phospholipids. This method has the particular advantage of separating PA with good resolution for assay by phosphorus content or radioactive label. PI can also be quantitated but different polyphosphoinositides could not be distinguished.

Adrenaline has been shown to increase ^{32}Pi incorporation in PI and PA of the rabbit iris muscle (Abdel-Latif, 1974), while ADP causes a decrease in phosphatidylinositol-4,5-bisphosphate associated also with increased turnover of PA(Vickers et al.,1982). The results reported in Chapter 7 are inconclusive but certainly indicate that adrenaline behaves in a different manner to

either ADP or thrombin with respect to phospholipid metabolism. The preliminary results obtained indicated that, when adrenaline potentiates ADP-induced aggregation, increased turnover of PI and PA is difficult to observe. Previous Chapters indicated that BPB may inhibit diacylglycerol kinase but this was not supported, since the amount of PA and the turnover of PA did not alter in the presence of BPB. In the presence of BPB, however, adrenaline and ADP seemed to increase incorporation of ^{3}H-arachidonic acid in PI, without increasing the amount of PI. Further investigation of PI metabolism with respect to changes among the different inositide classes should be undertaken. In the presence of BPB an exaggeration of the adrenaline effects on PI cycling may occur. Some evidence for the involvment of calcium in the potentiation mechanism has been presented in this thesis, and if, indeed, adrenaline does not alter PI metabolism in the initial stages of platelet interaction, then calcium mobilization could occur independently of the PI-effect.

8.2 CONCLUSIONS

1. With low concentrations of heparin as anticoagulant, adrenaline can cause potentiation of aggregation *in vitro* at concentrations which can be found in the circulation, supporting the theory that adrenaline plays a role in facilitating platelet activation at sites of vascular injury.

2. The calcium chelating effects of citrate are not a prerequisite for prostaglandin-dependent aggregation due to adrenaline. Citrate indeed may reduce the potentiating effects of both vasopressin and adrenaline, indicating the importance of calcium availability.

3 There must be at least two mechanisms by which adrenaline can potentiate platelet aggregation, firstly by causing increases in cyclooxygenase activity, and secondly, by a cyclooxygenase-independent mechanism, probably both involving α_2-receptor interaction. The cylcooxygenase-dependent pathway is the more efficient mechanism of adrenaline potentiation.

4. In the presence of certain phospholipase A_2 inhibitors, namely BPB and TPCK, adrenaline increases TXB_2 formation due to ADP, possibly due to increased release of arachidonic acid. This increased release, via a non-phospholipase A_2 pathway, may be due to stimulation of diacylglycerol lipase by adrenaline.

5. The immediate post-receptor events in the adrenaline response, may involve changes in phospholipids caused by other agonists and in particular, changes in the PI cycle. Adrenaline may influence diacylglycerol production or metabolism by ADP and thus influence a calcium-independent pathway via protein kinase C.

6. Further examination of the effects of dibenamine on adrenaline activation may reveal any possible influence of adrenaline on membrane calcium distribution independent of the PI-cycle.

7. The response to exogenous phospholipase C does not mimic the response to endogenous PI-specific phospholipase C. This may be because outer leaflet PC, the substrate for exogenous phospholipase C, does not participate in the cycling series of reactions. Nevertheless, the rapid formation of diacylglycerol by exogenous phospholipase C could be exploited for examining the importance of this compound.

8. Since phospholipase C induced aggregation in the presence of BPB occurs with an increased production of TXB_2, it is tempting to believe that diacylglycerol lipase is active while other routes for diacylglycerol metabolism are inhibited by BPB. This could not however, be confirmed with thrombin-induced aggregation.

9. The indication that adrenaline promotes diacylglycerol formation, but not PA formation should be examined further with respect to polyphosphoinositides. Changes in phospholipids due to adrenaline as measured by paper chromatography are difficult to observe.

10. The evidence from other workers that adrenaline by itself does not influence PI turnover, nor calcium uptake, in the initial interaction with platelets, should not deter investigation of these possibilities in relation to the mechanism of adrenaline potentiation of other agonists.

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