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Presence of Myristoylated Alanine Rich C Kinase Substrate (MARCKS) in Platelets and its Role in Secretion.

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ABSTRACT

It has been suggested that during platelet aggregation, actin polymerizes and the content of the secretory granules is released to the cell exterior. On the other hand, previous experiments from our laboratory suggest that actin disassembly, perhaps at a specific site, is required for platelet secretion (Marcu M.G., Zhang L., Nau-Staudt K. and Trifaró J-M, *Blood,* 1996, 87: 20-24; Marcu M.G., Zhang L., Elzagallaai A and Trifaró J-M., *J. Biol. Chem.* 1998, 273: 3661-3668). Stimulation of platelets by phorbol 12-myristate 13-acetate (PMA) induced pleckstrin phosphorylation and platelet aggregation and secretion. Inhibition of protein kinase C (PKC) is accompanied by inhibition of pleckstrin phosphorylation and platelet serotonin secretion. Here, we demonstrate the presence of myristoylated alanine-rich C kinase substrate (MARCKS), another PKC substrate, in platelets and its phosphorylation during PMA or thrombin stimulation. MARCKS is known to bind actin and cross-link actin filaments and its actin cross-linking activity is inhibited by PKC-induced phosphorylation of the protein. Both MARCKS phosphorylation and serotonin release from permeabilized and intact platelets stimulated by either PMA or thrombin, respectively, have the same concentration-dependency and were blocked by a peptide (MPSD) with the amino acid sequence corresponding to the phosphorylation site domain of MARCKS. Under these conditions, pleckstrin

and myosin light chain phosphorylation in response to either PMA or thrombin simulation was not modified. A similar peptide (Ala-MPSD) in which the four serine residues of MPSD were substituted by alanines was ineffective. The present results provide the first evidence that MARCKS may play a role in platelet secretion. Moreover, the fact that pleckstrin phosphorylation has a different concentration-dependency than that of MARCKS or serotonin release and was not modified when MARCKS phosphorylation and serotonin release were inhibited, suggests that pleckstrin is either not directly involved in platelet secretion or it might only be involved upstream in the cascade of events leading to exocytosis.

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Abbreviations.

AA:	arachidonic acid.
ABP-280:	actin-binding protein-280.
Ala-MPSD:	an MPSD peptide with its four serine residues replaced by alanine residues.
ATP:	adenosine 5'-triphosphate.
βARK:	β-adrenergic receptor kinase.
CaM:	calmodulin.
CD41a:	platelet antigen (receptor for fibrinogen).
DAG:	*sn*-1,2-diacylglycerol.
DNase I:	deoxyribonuclease.
DTS:	dense tubular system.
ECL:	enhanced chemiluminescence.
EDTA:	ethylenediamene tetraacetic acid.
EGTA:	glycol-bis(β-aminoethyl ether)N,N,N',N'-tetraacetic acid.
GP IIb/IIIa:	glycoprotein IIb/IIIa complex.
HEPES:	N-[2-hydroxyethyl]piperazine-N'-[2-ethanesulfonic acid].
[^{3}H]5-HT:	tritium-labelled serotonin.
IgG:	immunoglobulin G.
I.P.:	intact platelets.
IP_3:	inositol 1,4,5-triphosphate.
MARCKS:	myristoylated alanine rich C kinase substrate.
MEG-01:	a megakaryoplastic cell line established from the bone marrow of a patient with blast crisis of philadelphia (ph^1) chromosome-positive chronic myelogenous leukemia (Ogura et al, 1985).
MLC:	myosin light chain.
MPSD:	MARCKS phosphorylation site domain.
OCS:	open canalicular system.
PBS:	phosphate-buffered saline.
PI:	phosphatidylinositol.
PI 3-K:	phosphatidylinositol 3-kinase.
PIP:	phosphatidylinositol 4-monophosphate.
PIP_2:	phosphatidylinositol 4,5-bisphosphate.
PIP_3:	phosphatidylinositol 3,4,5-triphosphate.
PKC:	protein kinase C.
PMA:	phorbol 12-myristate 13-acetate.
P.P.:	permeabilized platelets.
[^{32}P]Pi:	inorganic phosphorus isotope.
PtdCho:	phosphatidylcholine.
PtdIns:	phosphatidylinositol.

PtdSer:	phosphatidylserine.
RACK:	receptors for activated C kinase.
SDS:	sodium dodecyl sulfate.
SDS-PAGE:	sodium dodecyl sulphate-polyacrylamide gel electrophoresis.
TCA:	trichloroacetic acid.
TEMED:	tetramethylethylene diamine.
vWF:	von Willebrand factor.

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Chapter (I)
Introduction

PART ONE:

Platelet Structure and Function.

Platelets, disc shaped subcellular fragments of megakaryocytes found circulating in the blood of all mammals, are chiefly known for their role in blood coagulation. These fully differentiated small cells have no nuclei or DNA but they contain active enzymes and mitochondria and have the complete machinery for cell movement and exocytosis (White, 1994; Hartwig, 1998). While circulating in the blood stream, platelets are frequently squeezed by other bigger blood cells against the surface of vessel endothelial lining, yet they still maintain their resting state and they do not interact with the contacting surface (Hartwig, 1998). However, once they are exposed to any vascular damage or challenged by any activating agent released by damaged endothelial cells, platelets can exhibit very different behavior and undergo a repertoire of morphological and biochemical changes designed to prevent hemorrhage. Platelet activation is a precisely controlled process and more than a dozen of signal transduction pathways are involved in this fascinating system (Siess, 1989; Colman et al, 1994). The process starts by the adherence of the platelets to the injured vascular surface through the exposure of subendothelial layers which are rich in adhesion proteins such as collagen, von Willebrand factor and fibronectin (Siess, 1989). Platelets then change from discoid to spherical shape and develop membrane projections known as pseudopods (Seiss, 1989). At this stage, primary reversible

aggregation can occur which is followed by the release reaction and then the secondary irreversible aggregation (Siess, 1989). The state of platelets being either in the resting or activated form is very important for survival; therefore, they are always kept under balance by activating and inactivating factors. Activation of platelets at the inappropriate time, or place, can lead to thrombosis with the development of the consequent life threatening stroke and myocardial infarction (Colman et al, 1994). On the other hand, a failure in platelet activation during vascular injury can lead to hemorrhage which, if not controlled, can cause serious health problems. Therefore, a complete understanding of the properties of platelets and their behavior is essential for the understanding of those fatal pathological conditions and their treatments.

This chapter concisely describes the structure of resting and activated platelets with a special focus on the role of filamentous actin (F-actin) in platelet activation. It also reviews the current knowledge of the platelet protein kinase C (PKC) enzyme family and their substrates in platelets. Different platelet permeabilization techniques and their advantages and disadvantages are also described in this chapter.

A. Structure of resting platelets.

Platelets circulate in blood as small discoid cells having a volume of 7.1 ± 4.6 μm^3, a diameter of 3.6 ± 0.7 μm and thickness of 0.9 ± 0.3 μm (Frojmovic and

Panjwani, 1976). In scanning electromicrographs, platelets appear as flat discs with a smooth featureless surface except of some random pits which represent the entrance to the open canalicular system (OCS) (Stenberg and Hill, 1999). The OCS is a series of invagination in the cell membrane making channels throughout the cytoplasm (Stenberg and Hill, 1999). This unique feature in platelet morphology gives platelets a sponge-like appearance and increases the area of contact between platelets and their surroundings. Development of new ways of fixation (White, 1983) has greatly increased our knowledge of the internal morphology of platelets and their ultrastructure (Fig 1.1) (Bentfeld-Baker and Bainton, 1982).

1. Exterior coat or glycogalyx.

A glycoprotein-rich exterior coat covers the membrane lipid bilayer of platelets (Stenberg and Hill, 1999). The platelet glycogalyx is thicker (15-20 nm) and more concentrated than that of other blood cells (Stenberg and Hill, 1999). It contains glycoproteins, glycolipids, mucopolysaccharides and adsorbed plasma proteins (Stenberg and Hill, 1999). The high content of carbohydrate and sialic acid residues in the platelet glycogalyx produces surface net negative charges which minimize attachment of platelets to each other during circulation. In addition, platelet surface glycoproteins serve as receptors for a wide variety of specific and nonspecific stimuli (White, 1994). Many of these glycoproteins are part of the integrin superfamily of

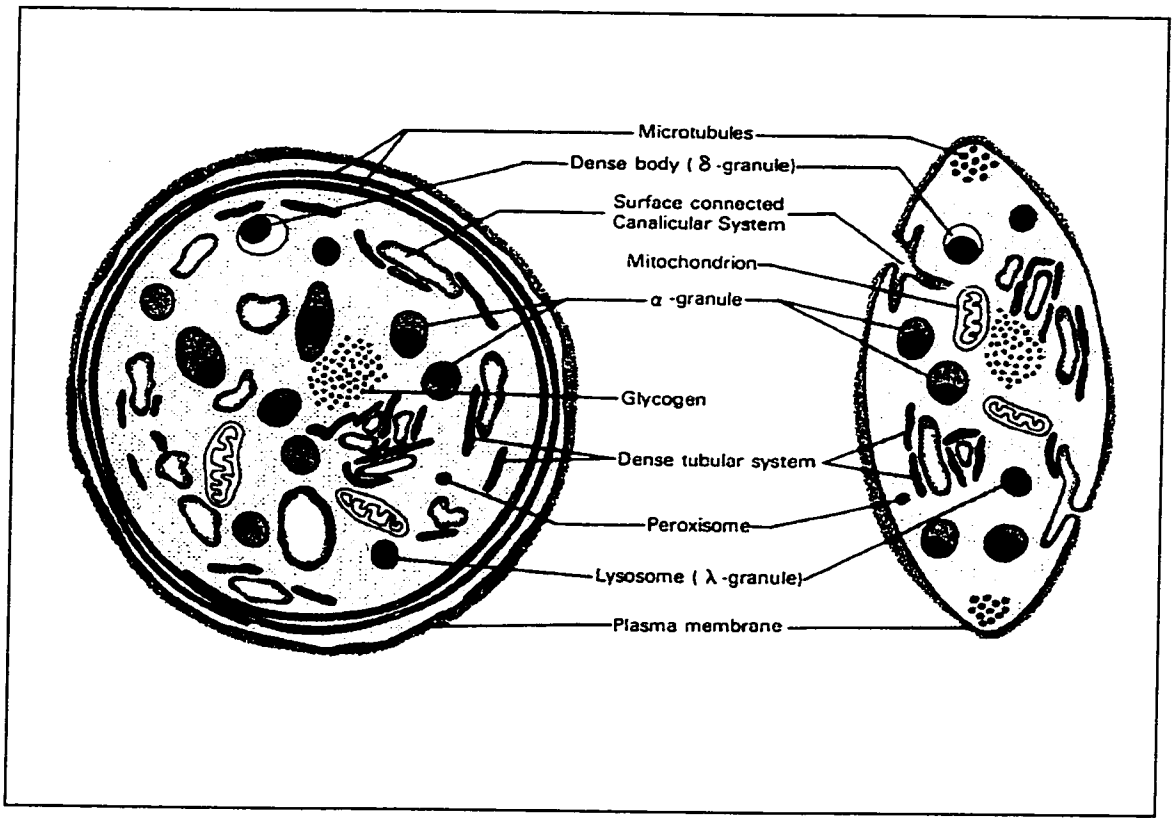

Fig 1.1. Structure of a resting platelet. Diagram of a resting human platelet showing components visible by electron microscopy and cytochemistry. (from Bentfeld-Barker and Bainton, 1982)

surface receptors (White, 1994). These receptors are involved in platelet adhesion and aggregation (Peerschke and López, 1998).

2. Platelet plasma membrane.

Similar to all cell membranes, the platelet plasma membrane consists of a double layer of glycolipids and phospholipids (Peerschke and López, 1998). Cholesterol represents an important element in the composition of platelet plasma membrane (Peerschke and López, 1998). Cholesterol is present in both the inner and outer leaflets of the membrane with the outer leaflet containing double the amount of cholesterol present in the inner leaflet (Peerschke and López, 1998). Membrane cholesterol content maintains the membrane fluidity and influences its transport and permeability (Peerschke and López, 1998). The platelet membrane also contains proteins components that are embedded in the lipid bilayer (Schick, 1994). These are mostly transmembrane receptors that convey signals from the cell exterior to the cytoplasm (Schick, 1994). The lipid/protein ratio in the platelet membrane is 0.58 (Schick, 1994). Receptors present on the surface of the platelet plasma membrane include: a) heptahelical transmembrane receptors such as thrombin receptors, ADP receptors, thromboxane A_2 receptors, α_2-adrenergic receptors and serotonin receptors (Peerschke and López, 1998); b) platelet adhesion receptors which include adhesion molecules of the integrin family, such as the fibrinogen receptor (GP IIb-IIIa), the vitronectin receptor, the fibronectin receptor (GPIc-IIa), the laminin receptor, the

collagen receptor (GPIa-IIa) and the von Willebrand factor receptor (GP Ib-IX-V complex) (Peerschke and López, 1998); adhesion receptors of the selectin gene family such as P-selectin and adhesion receptors of the immunoglobulin gene family such as PECAM-1 and PCAM-2 (Peerschke and López, 1998); c) platelet Fc receptors (FcγRIIA) (Peerschke and López, 1998); d) other platelet receptor such as glycoprotein IV (Peerschke and López, 1998), which acts as a receptor for the α-granule protein thrombospondin (Peerschke and López, 1998), and glycoprotein VI which is thought to bind to collagen (Peerschke and López, 1998).

i. Thrombin receptors.

Platelets have three classes of thrombin binding sites: low affinity binding sites (590,000 sites/platelet; K_d ~2900 nM), moderate affinity binding sites (1700 sites/platelet; K_d ~11 nM), and high affinity binding sites (50 sites/platelet; K_d ~0.3 nM) (Harmon and Jamieson 1986). The cloned thrombin receptor is a 425 amino acid protein which belongs to the family of G-protein coupled serpentine receptors (Brass et al, 1994). It has an extended extracellular N-terminus containing the site for thrombin-induced cleavage (Fig 1.2), seven hydrophobic domains which represent the seven transmembranous domains, and an intracellular C-terminus containing sites for posttranslational modifications including palmitoylation (Brass et al 1994). The C-terminus also contains six serine residues which serve as substrates for β-adrenergic receptor kinase (βARK) (Brass et al, 1994). The C-terminus also contains

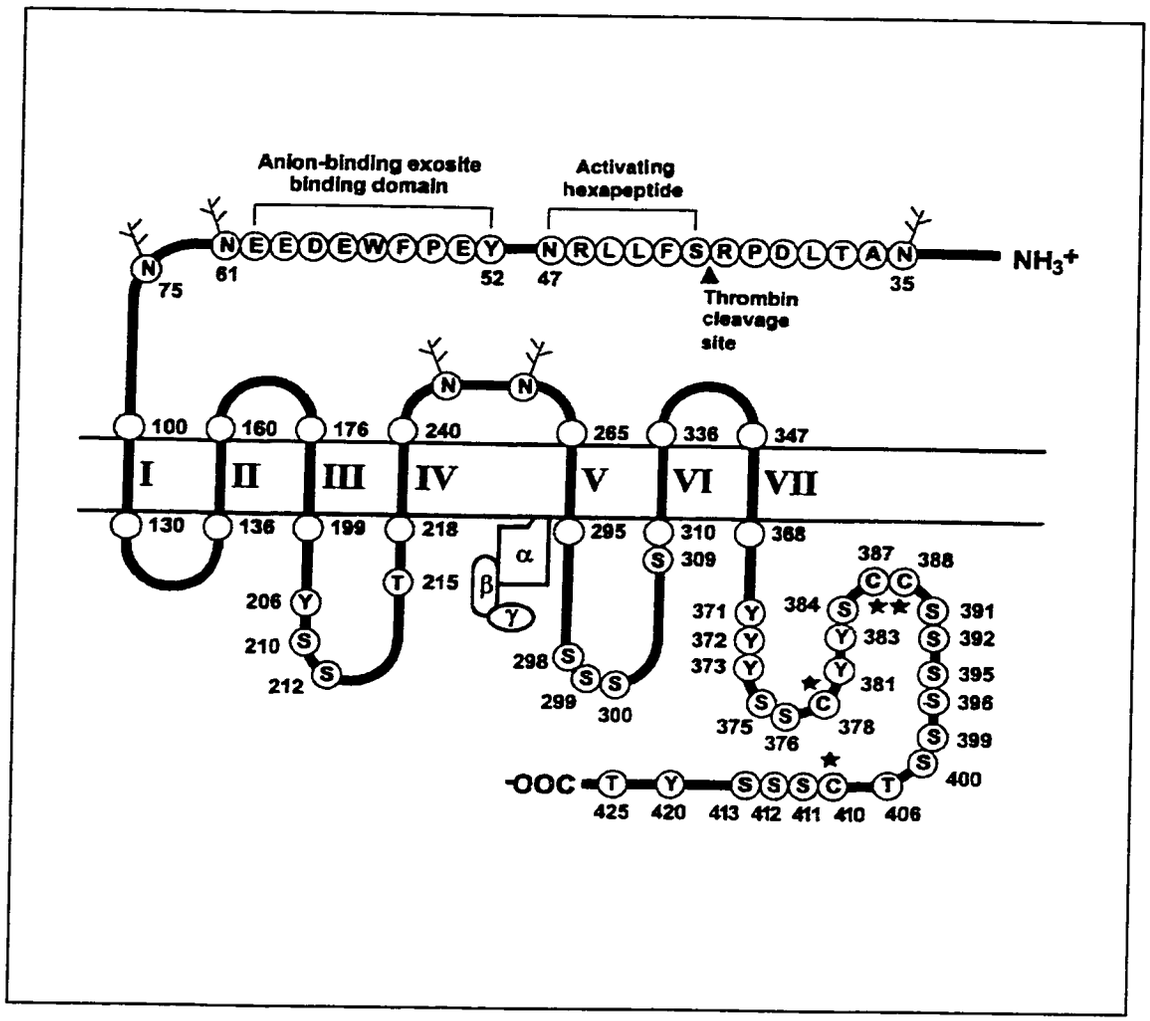

Fig 1.2. Structure of the thrombin receptor (from Kroll and Sullivan, 1998)

phosphorylation sites for protein kinase C (PKC) and protein kinase A (PKA) (Brass et al, 1994). These phosphorylation sites are thought to be involved in desensitization and down-regulation of the receptor (Brass et al, 1994). Activation of thrombin receptor is unique among other G-protein coupled receptors in that it involves proteolytic cleavage of the receptor N-terminus by thrombin (Kroll and Sullivan, 1998). This leads to exposure of a tethered ligand peptide that activates the receptor. Supporting this hypothesis is the fact that a 14-residues peptide corresponding to residues Ser^{42} to Phe^{55} of the thrombin receptor was found to cause platelet aggregation (Brass et al, 1994).

Activation of thrombin receptors leads to the activation of membrane bound phospholipase C (PLC) and the consequent hydrolysis of inositolphospholipids, i.e., phosphatidylinositol 4,5-bisphosphate (PIP_2) (Kroll and Sullivan, 1998). This event is one of the earliest measurable reactions in thrombin-stimulated platelets (Lapetina, 1990). The thrombin receptor is linked to PLC through two heterotrimeric G-proteins: a pertusis toxin-sensitive G_p and a pertusis toxin-insensitive G_q protein. Thrombin also inhibits adenylate cyclase through a pertusis toxin-sensitive G_i protein (Kroll and Sullivan, 1998). Inositol phospholipids hydrolysis results in production of two important second messengers: *sn*,1,2-diacylglycerol (DAG) and inositol 1,4,5-triphosphate (IP_3) which activate protein kinase C and mobilize cytosolic calcium, respectively (Kroll and Sullivan, 1998).

Exhaustion of the cellular reservoir of phosphoinositol phospholipids triggers

the activity of series of inositol kinases to refill the depleted pool (Lapetina, 1990). Novel phosphoinositol species that are generated in platelets in response to thrombin stimulation are the 3-inositol phospholipids (Lapetina, 1990). These are produced by phosphorylation of phosphoinositides at the 3-position of the inositol ring by the action of phosphatidylinositol 3-kinase (PI 3-K) (Lapetina, 1990). Such inositol phospholipids are not substrates for PLC and they may serve as cellular second messengers that mediate platelet responses (Lapetina, 1990). Wortmannin (Ui et al, 1995) and LY294002 (Vlahos et al, 1994), two specific inhibitors of PI 3-K, were shown to inhibit platelet aggregation (Zhang and Rittenhouse, 1995) and secretion (Yatomi et al, 1992, Oda et al, 1997 and Chasserot-Golaz et al, 1998). Thrombin stimulation of platelets also results in the activation of the arachidonic acid (AA) metabolism pathway. Liberation of AA occurs through activation of phospholipase A_2 (PLA_2) as a consequence of the increase in the cytosolic concentration of free Ca^{2+} (Lapetina, 1990). However, regulation of PLA_2 in platelets is not fully understood. A major metabolite of the platelet AA pathway is thromboxane A_2 which is itself a very potent platelet agonist (Lapetina, 1990). Thromboxane A_2 is formed by action of the enzymes cyclooxygenase and thromboxane A_2 synthase (Lapetina, 1990).

3. Submembrane (cortex) region.

It is the area lying just beneath the platelet plasma membrane and has special features that differentiate it from other areas of the cytoplasm. This area contains an

actin and spectrin-based cytoskeletal network which probably acts, in resting platelets, as a barrier to platelet organelles preventing them from reaching the plasma membrane. It has been suggested that the function of these submembrane actin filaments might be to act, along with the peripheral microtubule coils, to maintain the discoid shape of platelets (White, 1994).

4. Platelet organelles.

In addition to secretory granules, platelets contain at least three different types of intracellular organelles. Mitochondria are the easiest to identify using electron microscopy. Other organelles which are identifiable using some specific cytologic staining procedures are lysosomes and peroxisomes (Hartwig, 1998; Stenberg and Hill, 1999). They are vesicles which contain enzymes that function in the degradation of materials ingested by pinocytosis or phagocytosis (Hartwig, 1998).

5. Platelet granules.

Platelets contain two types of secretory granules: α-granules and dense granules. Lysosomes and microperoxisomes are also considered as granules by some investigators (Stenberg and Hill, 1999). Alpha-granules are the most abundant type of granule present in human platelets (Stenberg and Hill, 1999). They usually assume a round or oval shape, and have a diameter of 300-500 nm. Dense granules have a

diameter of 200-300 nm and appear in thin section electomicrographs with a very characteristic bull's eye appearance (Stenberg and Hill, 1999). The number of dense granules present in a platelet is proportional to its serotonin content (Zucker-Franklin, 1997) Alpha-granules contain soluble adhesive proteins (e.g., fibrinogen, fibronectin, thrombospondin and von Willebrand factor), enzymes and inhibitors (e.g., plasminogen and α_2-antiplasmin), procoagulants (e.g., factor V and PF4), growth factors (e.g., PDGF, TGF-α, TGF-β, GGF and ECGF), membrane bound adhesive proteins (e.g., P-selectin), bacteriocidal (e.g., β-lysin) and other (e.g., Mg^{2+} and guanine nucleotide) (Hartwig, 1998); whereas dense granules contain neurotransmitter-like agents (e.g., ADP, ATP, serotonin and guanine nucleotides), membrane bound adhesion proteins (e.g., P-selectin and granulophysin) and other (e.g., Ca^{2+} and pyrophosphate) (Hartwig, 1998). Exocytosis in platelets is thought to be unique among that of other cell types, thus, it has been given the term "platelet release reaction" distinguishing it from the exocytosis process in other cells (Sherry, 1974; Hartwig, 1998). It has been suggested that the granules fuse with the membrane of the OCS instead of the plasma membrane and discharge their contents into the OCS in the center of the activated platelet (Hartwig, 1998). Although this theory is the most widely accepted, some published experimental data has suggested other possibilities (see secretion section, Ginsberg et al, 1980; Polasek et al, 1987; Zucker-Franklin et al, 1995; Marcu et al, 1996; Elzagallaai et al, 1998; Elzagallaai et al, 2000).

6. Membrane compartments.

i. Open canalicular system.

The open canalicular system (OCS) is an extensive system of membrane bound channels wandering through the platelet cytoplasm giving platelet sections an appearance of a sponge (Hartwig, 1998). It serves as a way system for the cell in one of its functions. That is to say, it acts as a passage into which secretory granule contents are released during the platelet secretion (Hartwig, 1998). Another function of the OCS is a storage site for membrane receptors. These can be moved between plasma membrane and OCS membrane upon platelet activation (Wencel-Drake, 1990). In addition, the platelet OCS can be a source of redundant cell membrane for cell spreading (Hartwig, 1998).

ii. Dense tubular system.

The dense tubular system (DTS) is a membrane compartment present in the platelet cytoplasm which has the same function of the smooth endoplasmic reticulum in other cells (Hartwig, 1998). It serves as a Ca^{2+} storage system and is equipped with Ca^{2+}-pumps (Ca^{2+}/Mg^{2+} ATPase) that maintain the free Ca^{2+} concentration of the cytosol of resting platelets in the nanomolar range (Hartwig, 1998). The DTS contains receptors for the second messenger inositol 4,5-triphosphate (IP_3) which, as indicated above, is generated from the hydrolysis of phospholipids during platelet activation (Kroll and Sullivan, 1998). The DTS is also a site for prostaglandins and

thromboxanes synthesis (Hartwig, 1998).

iii. Cytoplasmic inclusions.

The only ones present in platelets are glycogen particles which can be observed clearly in the electronmicrographs of platelet sections (Stenberg and Hill, 1999).

B. The platelet cytoskeleton.

Under light microscopy platelet cytoplasm appears clear and transparent (White, 1994). Because of this, the term "hyaloplasm" or "hyalomare" was used to describe the platelet matrix (White, 1994). However, electromicroscopy has revealed the complexity of the platelet cytoplasmic structure and its transformation during platelet activation (White, 1994). The terms "cytoskeleton," "cytomatrix" or "sol-gel zone" refer to the detergent resistant elements of the cytoplasm of all cells including platelets (White, 1994). Most of the information regarding the organization of the platelet cytoskeleton has come from biochemical studies using detergent (Triton X-100) lysed platelets (Fox, 1993). Lysates are then subjected to differential centrifugation to separate fragmented actin filaments and soluble components from the highly cross-linked filaments (Fox 1993). Under these conditions, highly cross-linked actin filaments can be sedimented at relatively low g-forces (Fox, 1993). Combining this technique with SDS-PAGE and immunoblotting provided a great deal of information about proteins that are involved in the organization of the platelet

cytoskeleton (Fox, 1993). In addition, the use of electronmicroscopy, and the improved fixation and staining techniques that permit the preservation of the platelet cytoskeleton morphology have increased our understanding of the changes that take place in the organization of actin filaments during platelet activation (Hartwig, 1998). Based on such techniques platelet cytoskeleton can be divided into three distinct components: the membrane skeleton, the peripheral microtubule coil system and the cytoplasmic actin filaments network (Hartwig, 1998).

1. The membrane skeleton.

The membrane skeleton is a scaffold of cytoskeletal proteins which make up a two-dimensional network lining the cytoplasmic face of the lipid bilayer (Hartwig, 1998). It is composed of long spectrin tetramers that are interconnected by actin filaments (Hartwig, 1998). This network is strengthened by the actin binding protein ABP-280 which binds to the sides of cytoplasmic actin filaments and to the membrane bound $GPIb_{\alpha\beta}$-IX-V (Hartwig, 1998, Fig 1.3). Glycoprotein IIb-IIIa and glycoprotein Ib-IX are major platelet membrane glycoproteins that have been shown to be involved in stabilizing the membrane skeleton and assuring its association with the plasma membrane (Stenberg and Hill, 1999). Glycoprotein IIb-IIIa acts as a receptor for fibrinogen and association of this glycoprotein with the membrane skeleton is thought to represent a connection between cytoskeletons of adhered platelets; thus mediating aggregation and retraction of the platelet clot (Hartwig,

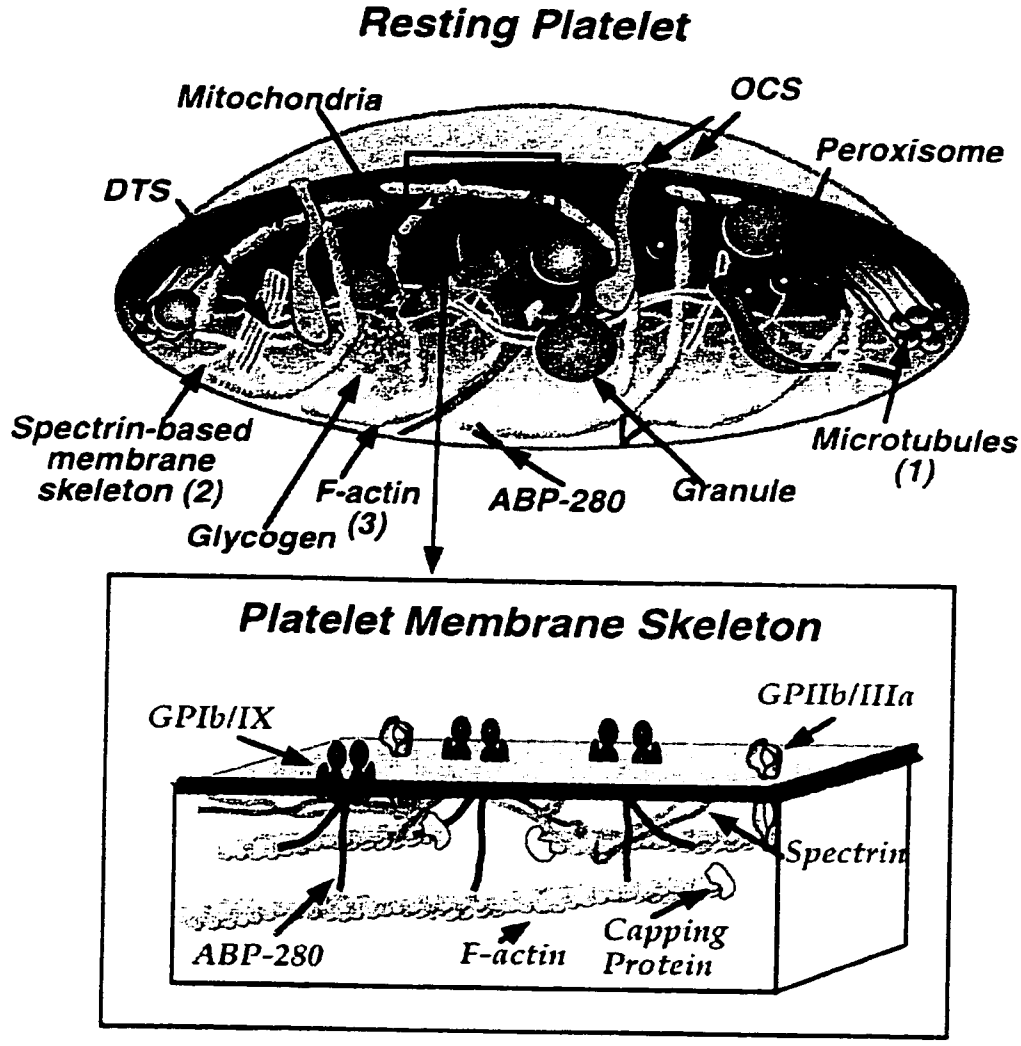

Fig 1.3. Schematic diagram showing the structural features of a resting platelet and its membrane skeleton. The discoid shape of resting platelets is maintained by three cytoskeletal structures: (1) a marginal microtubule coil, (2) a spectrin based membrane skeleton, and (3) a rigid network of cytoplasmic actin filaments (from Hartwig, 1998).

1998). In platelet detergent lysates, the membrane skeleton is viewed as a continuous structure at the edges of the cytoplasmic actin filaments (Fox, 1993). In experiments performed using Triton X-100 lysed platelets, other actin binding proteins such as α-actinin, talin, vinculin, $pp60^{c\text{-}src}$, $pp62^{c\text{-}yes}$ and $p21^{ras}$ were found to be associated with the membrane skeleton (Fox et al, 1988). Therefore, these proteins might be involved in the regulation of the membrane skeleton in both resting or activated platelets (Fox, 1993).

2. The peripheral microtubule coil.

In cross sections of fixed platelets microtubules appear as a group of 8 to 24 circular forms, having a diameter of ~25 nm each (Hartwig, 1998). Each microtubule consists of 12-15 subfilaments (Hartwig, 1998). Each profilament is a linear stack of α and β tubulin subunits (Hartwig, 1998). Microtubules are present at the periphery of the cell along the thin axis of the resting discoid platelet (Fig 1.1 and 1.3, White, 1994). This suggests that circumferential microtubules are involved in supporting the discoid shape of resting platelets (Hartwig, 1998). Further evidence that support this hypothesis is the concomitant loss of the discoid shape and the peripheral microtubule coil in platelets incubated at low temperature and the restoration of both features when platelets were warmed up to 37°C (White, 1994). Chemical agents that disassemble microtubules such as colchicine, vincristine and vinblastine also have the same effect on microtubules and consequently platelet shape (White, 1994).

Microtubules have another hypothesized role in platelet function, that is the mediation of platelet central cytoplasmic contraction during aggregation (White, 1994). However, more recent studies using Taxol, a macrocyclic alkaloid that prevents microtubule disassembly, have proven that microtubules disassembly and relocation are not necessary in any platelet function including the contraction of the central gel with centralization of organelles (White, 1994; Zucker-Franklin, 1997).

3. Cytoplasmic actin network.

Concentration of actin in platelets is approximately 0.5 mM exists in equilibrium between polymerized (F-actin) and non-polymerized (G-actin) actin (Hartwig, 1998). In resting platelets, 40% of actin is in the polymerized form making up about 2000 filaments of an average length of approximately 1.1 μm (Hartwig, 1998). Actin polymers are double helical polarized structures and the kinetics of the growth of an actin filament at its two ends is different in presence of ATP (Polard and Mooseker, 1981). The two ends of actin filaments have been defined as "barbed" and "pointed" ends as a result of decoration of the filaments with myosin subfragment 1 (Hartwig, 1998). Growth of actin filaments at their "barbed" ends is ten folds higher that at their "pointed" ends (Hartwig et al, 1995; Hartwig, 1998). The length of actin filaments in resting platelets is kept constant by two strategies: (a) Preventing filament growth at its pointed end by storing actin monomers as 1:1 complexes with proteins such as β-thymosin whose affinity for G-actin is higher than that of pointed filament

ends for actin monomers (Hartwig, 1998), and (b) sequestration of the filament barbed end by capping proteins such as gelsolin (Yin and Stossel, 1979), Cap Z (Isenberg et al, 1980) and scinderin (Rodriguez Del Castillo, 1992). Platelet activation and the subsequent increase in levels of intracellular free Ca^{2+} and other second messengers leads to changes in the intracellular environment with a shift of the equilibrium of the actin state (Hartwig, 1998).

In resting platelets, cytoplasmic actin filaments arise from the center of the cell and from there they radiate toward its periphery (Hartwig, 1998). When the filaments reach the plasma membrane they curve and run parallel to the membrane (Hartwig, 1998). The three-dimensional structure of the cytoplasmic actin network is maintained by the existence of cross-linking proteins such as spectrin, ABP-280 and α-actinin which bind to the sides of actin filaments and organize them into a higher order ultrastructure (Hartwig and Kwiatkowski, 1991). Another actin binding protein found in platelets is tropomyosin (Fox et al, 1988). There is some evidence that this protein may regulate the interaction of actin with other proteins (Fox, 1993).

C. Cytoskeleton Reorganization during platelet activation.

During platelet activation, its cytoskeleton undergoes dramatic reorganization due to cycles of actin assembly-disassembly which take place in different areas of the activated platelet (Hartwig, 1998). The amount of filamentous actin rapidly increases

from 30-40 % of total actin to 60-70 % during platelet activation (Hartwig, 1998). Actin assembly appears in at least two locations: the actin network at the cell periphery, and in the developing filopodia (Fox, 1993). The increase in actin assembly is generated by the uncapping of new actin barbed ends and by the liberation of actin monomers (Hartwig, 1998). This process was found to be Ca^{2+} dependent, and Ca^{2+}-dependent actin severing proteins such as gelsolin and scinderin seem to be involved in this process. On the other hand, experimental evidence has been gathered to suggest that actin disassembly takes place in certain areas during platelet activation to mediate specific (i.e., secretion) platelet responses (Marcu et al, 1996; Elzagallaai et al, 1998; Elzagallaai et al, 2000).

The cytoskeletal reorganization of activated platelets can be divided into two categories: those that occur independently of platelet aggregation, and those that occur in platelets which are let to aggregate (Fox, 1993). Shortly after platelet activation, actin-binding proteins including α-actinin, tropomyosin, ABP-280 and talin undergo relocalization to areas such as the cell periphery and developing filopodia (Hartwig, 1998). At the same time, a number of other cytoskeletal proteins such as talin, myosin, cortactin and other unidentified proteins become phosphorylated in activated platelets (Fox, 1993). Myosin is another very important cytoskeletal protein of platelets which associates with cytoplasmic actin filaments and is involved in filament organization during platelet activation (Stark et al, 1991). It is well established that phosphorylation of myosin induces its binding to actin filaments in order to form the

central contractile gel in activated platelets (Stark et al, 1991).

Platelet response to activating agents occurs in a series of successive events including adhesion, shape change, secretion and aggregation. Depending on the condition in which activated platelets are, they may exhibit all or some of these responses. Each of these responses is perhaps mediated by separate signal transduction pathways. The overlapping of these pathways and the complexity of the system make it extremely difficult to study individual responses in a condition when dramatic changes in platelet architecture are taking place. Cytoskeletal reorganization seems to mediate a great part, if not all, of platelet responses. However, each response is likely to occur as a result of a specific and highly localized rearrangement of the platelet cytoskeleton.

1. Platelet adhesion.

During vascular injury, platelets adhere to the exposed subendothelial surface through interaction with collagen and von Willebrand factor (vWF) (Siess, 1989). In this case adhesion occurs simultaneously and is augmented by platelet shape change which allows more contact between the platelet surface and the tissue. Platelets can also adhere to collagen fibers and artificial surfaces *in vitro*, although the mechanism involved may be different (Siess, 1989). Activation of platelets leads to exposure on the platelet surface of more adhesion receptors such as GP Ib-IX-V and GP IIb-IIIa (Peerschke and López, 1998). These molecules along with other secreted adhesion

proteins, such as fibrinogen and thrombospondin, mediate the adhesion of platelets to each other and the formation of the primary aggregates (Peerschke and López, 1998). The membrane skeleton is thought to be involved in the exposure and maintenance of the membrane adhesion receptors (Fox, 1993).

2. Shape change.

The first response to stimulation of platelets in suspension is shape change (Siess, 1989; Hourani and Cusack, 1991). Platelet shape change has two distinct features: change in platelet shape, from discoid to spherical, and projection of membrane bound spikes known as pseudopodia (Hartwig, 1998). These two morphological events are mediated solely by reorganization of the platelet cytoskeleton and can occur within seconds of platelet exposure to agonists (Hartwig, 1998). Platelet spheration requires disassembly and assembly of both actin filaments (Hartwig, 1998) and microtubules (White, 1994). On the other hand, formation of filopodia and lamellipodia is mediated by assembly of long bundles of actin filaments that support the new structure (Hartwig, 1998).

Elevation in the concentration of free cytosolic Ca^{2+} to a micromolar range is the main initiator of platelet shape change (Siess, 1989). Resting platelets maintain a concentration of free Ca^{2+} in their cytoplasm of 10-20 nM (Hartwig, 1998). Stimulation of platelets by an agonist like thrombin leads, through G-protein mediated

activation of phospholipase C (PLC), to the generation of inositol triphosphate (IP_3) (Lapetina, 1990). The latter causes liberation of Ca^{2+} from the endogenous stores by activation of IP_3 receptors (Lapetina, 1990). Release of Ca^{2+} from platelet endogenous stores is capable of elevating the cytosolic Ca^{2+} to 3-5 μM (Hartwig, 1998). In addition Ca^{2+} influx through receptor operated Ca^{2+} channels present in the cell membrane increase cytosolic Ca^{2+} within seconds to 1-2 μM (Siess, 1989). These two pathways can raise the concentration of free Ca^{2+} in the platelet cytoplasm to levels as high as 10 μM (Siess, 1989). Ca^{2+}-dependent actin severing proteins such as gelsolin and scinderin are activated at this concentration of free Ca^{2+} (Yin and Stossel, 1979; Rodriguez del Castillo et al 1990; Marcu et al, 1996). Severing of actin filaments would cause release of the constrain imposed on the membrane skeleton allowing plasma membrane to expand and flow outward and the cell to round (Hartwig, 1998). On the other hand, severing of actin filaments will also lead to creation of more barbed ends which would promote actin assembly and growth of the actin filaments which is necessary for formation of filopodia and lamellipodia (Hartwig, 1998). It is evident that growth of actin filaments takes place near the cytoplasmic face of the plasma membrane as a result of the generation of sufficient amount of potent phospholipids (i.e., PIP2, PIP_3). These phospholipids bind and inactivate capping proteins leading to uncapping of the barbed ends of actin filaments (Hartwig, 1998).

3. Aggregation.

Platelet aggregation, "*in vitro,*" requires at least two conditions: presence of extracellular free Ca^{2+} and platelet stirring (Nishikawa et al, 1980; Taylor and Heptinstall, 1980; Gear, 1981; Font et al, 1992; Lanza et al, 1992). At the same time, platelets stimulated with agonist such as thrombin can reach the maximum magnitude of secretion in absence of extracellular Ca^{2+} (Feinman and Detwiler, 1975; Haslam and Davidson, 1984) or preparation stirring (Nishikawa et al, 1980; Hashimoto et al, 1997). However, aggregation and platelet secretion are highly connected events because of two facts: 1) activated platelets secrete materials that can either induce or mediate platelet aggregation (Siess, 1989), and 2) platelet-platelet contact during aggregation can lead to activation of certain pathways that promote secretion of platelet granules (Fox, 1993; Law et al, 1999). Platelet dense granule deficiency (Hermansky-Pudlak syndrome) is associated with defective platelet aggregation *in vitro* and bleeding diathesis (Zucker-Franklin, 1997). A number of morphological and biochemical changes occur in aggregating platelets (Fox, 1993). These changes include cytoskeletal reorganization, tyrosine phosphorylation and calpain activation (Fox, 1993; Law et al, 1999). These changes seem to be mediated through platelet-platelet contact as they occur more rapidly when the platelet suspension is stirred (Fox, 1993). One of the aggregation-dependent events is the translocation of some cytoskeleton-associated proteins such as vinculin, talin, spectrin, pp60 $^{c\text{-}src}$, pp62 $^{c\text{-}yes}$

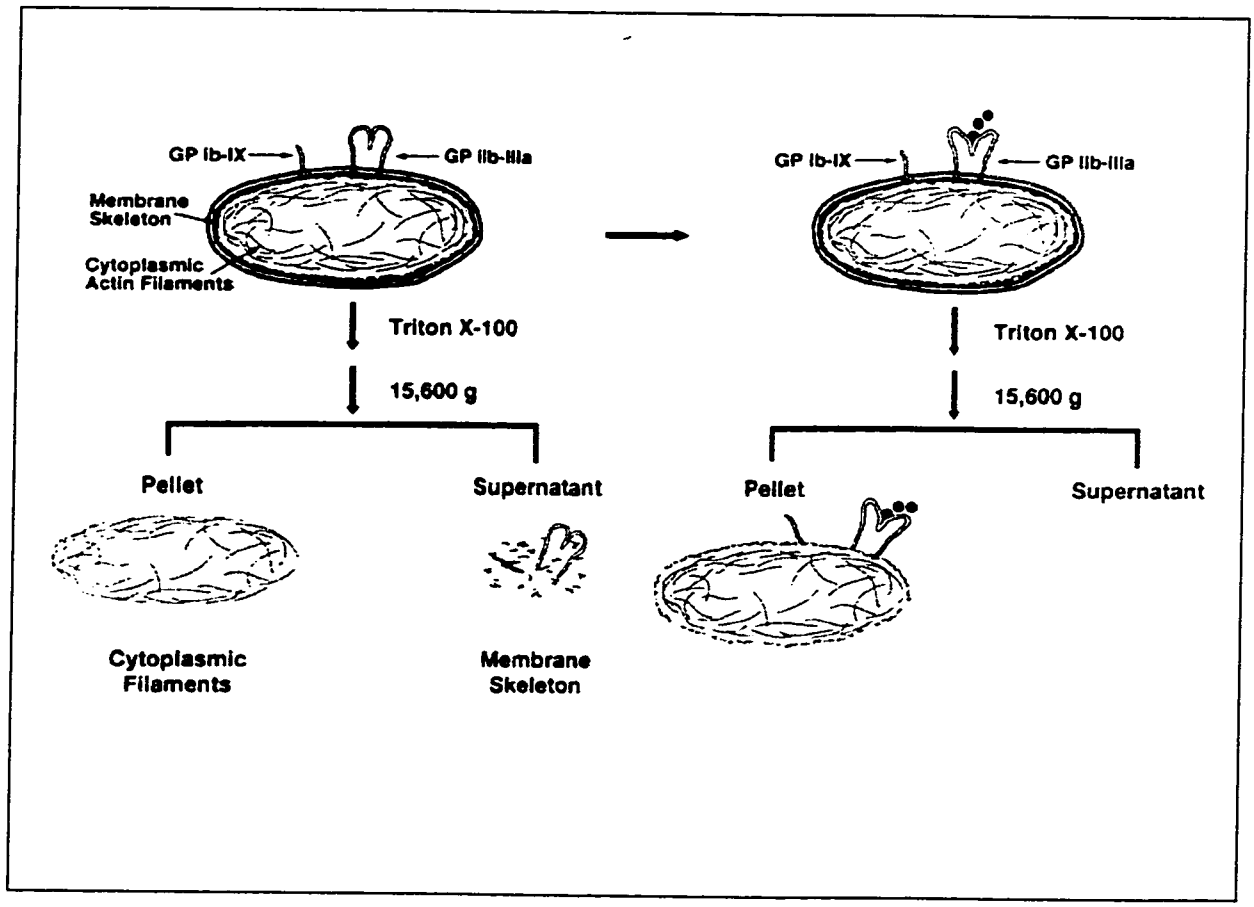

Fig 1.4. Schematic representation of the activation-induced redistribution of GP IIb-IIIa and membrane skeleton proteins from the low-speed to the high-speed detergent-insoluble fraction of platelets. **Left**: When unstimulated platelets are lysed with Triton X-1000 and the detergent lysates centrifuged at 15,600 g, cytoplasmic actin filaments are sedimented. The membrane skeleton remains in the 15,600 g supernatant. **Right**: When platelets are stirred with an agonist, the membrane skeleton becomes reorganized such that it can now be sedimented with cytoplasmic actin at 15,600 g. (From Fox, 1994).

and GAP to the highly cross-linked actin filaments fraction (Fox, 1993). This suggests that stirred platelets which are allowed aggregate are subjected to further cytoskeletal reorganizations that are different from those that occur during initial platelet activation. The membrane associated GP IIb-IIIa seems to be involved in this process (Fig 1.5). Cross-linking of GP IIb-IIIa receptor leads to generation of outside-in signals that cause more cytoskeletal rearrangement in aggregating platelets (Hartwig, 1998). Outside-in signaling through transmembranous integrin proteins can lead to cytosolic Ca^{2+} elevation and tyrosine phosphorylation of number of platelet proteins (Law et al, 1999).

4. Secretion.

The mechanism of platelet secretion has been for long subjected to controversial interpretation (Sherry, 1974; Ginsbberg et al, 1980; Carroll et al, 1982; Polsaek et al, 1987; Siess, 1989; Zucker-Franklin et al, 1995; Marcu et al, 1996; Marcu et al, 1998; Hartwig, 1998; Elzagallaai et al, 1998; Elzagallaai et al, 2000). The most popular and longest standing theory is what is known as "platelet release reaction" (Shery, 1976; Carroll et al, 1982; Siess, 1989; Hartwig, 1998). This hypothesis suggests that during platelet activation and formation of the contractile gel, platelet granules (i.e. secretory vesicles) become centralized and their content is extruded to the open canalicular system, which in turn, acts as a way to drain the

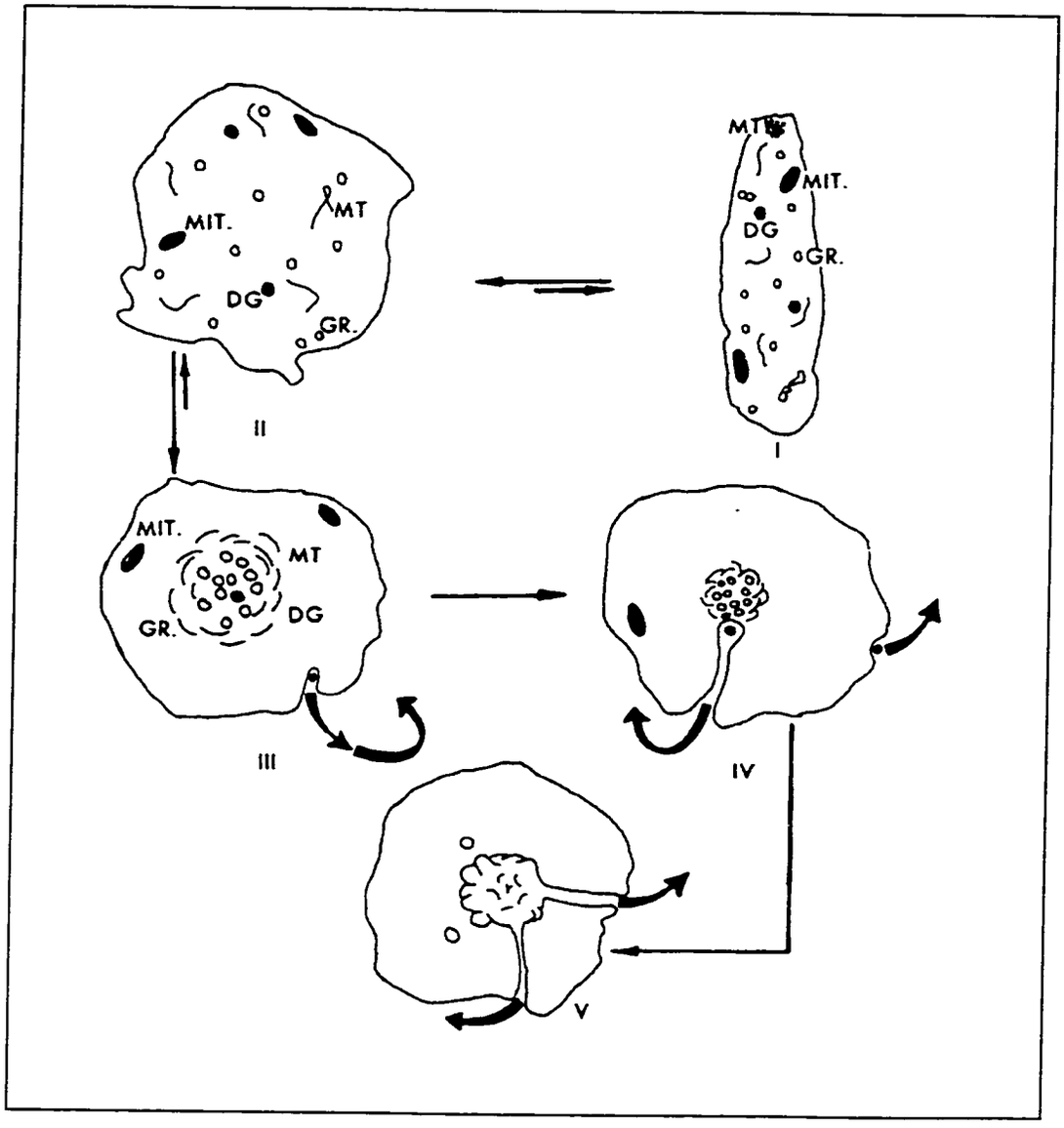

Fig 1.5. Platelet release reaction. Diagram showing the stages of the "platelet release reaction" as hypothesized. During platelet activation platelet contractile gel will squeeze platelet granules and extrude their content the open canalicular system (from Sherry, 1974).

secreted material to the cell exterior (Hartwig, 1998). This unique procedure is thought to occur only in platelets differentiating them in this aspect from any other cell type in the body (Sherry, 1976; Hartwig, 1998). However, this theory has been challenged by many findings including: (a) bovine platelets do not possess a surface connected canalicular system yet they still secret normally (Zucker-Franklin et al, 1995), (b) secretion of platelet factor 4 has been shown to be mediated through migration of secretory material to the platelet periphery (Ginsberg et al, 1980), (c) using electronmicroscopy and fixation procedures designed to preserve cell membrane and its associated structures, Polasek and colleagues (1989) could demonstrate that platelet secretion involves peripherization of platelet granules and their association with plasma membrane, and (d) experiments using recombinant scinderin, a Ca^{2+}-dependent actin severing protein, suggested that actin disassembly, perhaps at specific sites, mediate Ca^{2+}-induced secretion from platelets (Marcu et al, 1996; Marcu et al, 1998).

The morphology of platelets in clots is characterized by an elongated shape and the appearance of bundles of retracting fibers. The cell center becomes an electron dense mass because of the centralization of platelet organelles as a result of formation of the contracting gel (Hartwig, 1998). The contractile force causes rupture of cells and release of microvesicles (Hartwig, 1998). The final stages of platelet aggregation are so dramatic that platelets lose their defined shape and the whole clot becomes a

piece of interconnected tissue (White, 1994).

D. Protein kinase C (PKC) and platelet function.

1. Protein kinase C (PKC).

First discovered in bovine brain by Nishizuka and co-workers in 1977, protein kinase C (PKC) family has been extensively studied over the past two decades. This family of heterogenous serine/threonine protein kinases has been implicated in many biological processes including development, proliferation, neoplastic transformation, differentiation, apoptosis and neurotransmission. PKC is a family of multiple isozymes which may explain the diversity and complexity of the cellular function of PKC (Mellor and Parker, 1998). PKC consists of a single stretch of amino acid sequence having a molecular weight between 76 and 83 KDa with the exception of PKC ζ and λ which are 67 KDa and PKC μ which is 102 KDa (Quest, 1996). The structure of these enzymes can be divided into four highly conserved regions (C_1-C_4) which are separated by five variable regions (V_1-V_5) (Fig 1.6) (Liu and Heckman, 1998). The C_1 domain contains a sequence motif known as the pseudosubstrate which regulates the kinase activity by blocking the catalytic site (Quest, 1996). This domain also contains a cystine-rich region which consists of two zinc finger motifs which represent the binding site for DAG and phorbol esters (Quest, 1996). Calcium binding site lies in the C_2 region as PKC isozymes which lack this region exhibit Ca^{2+}-

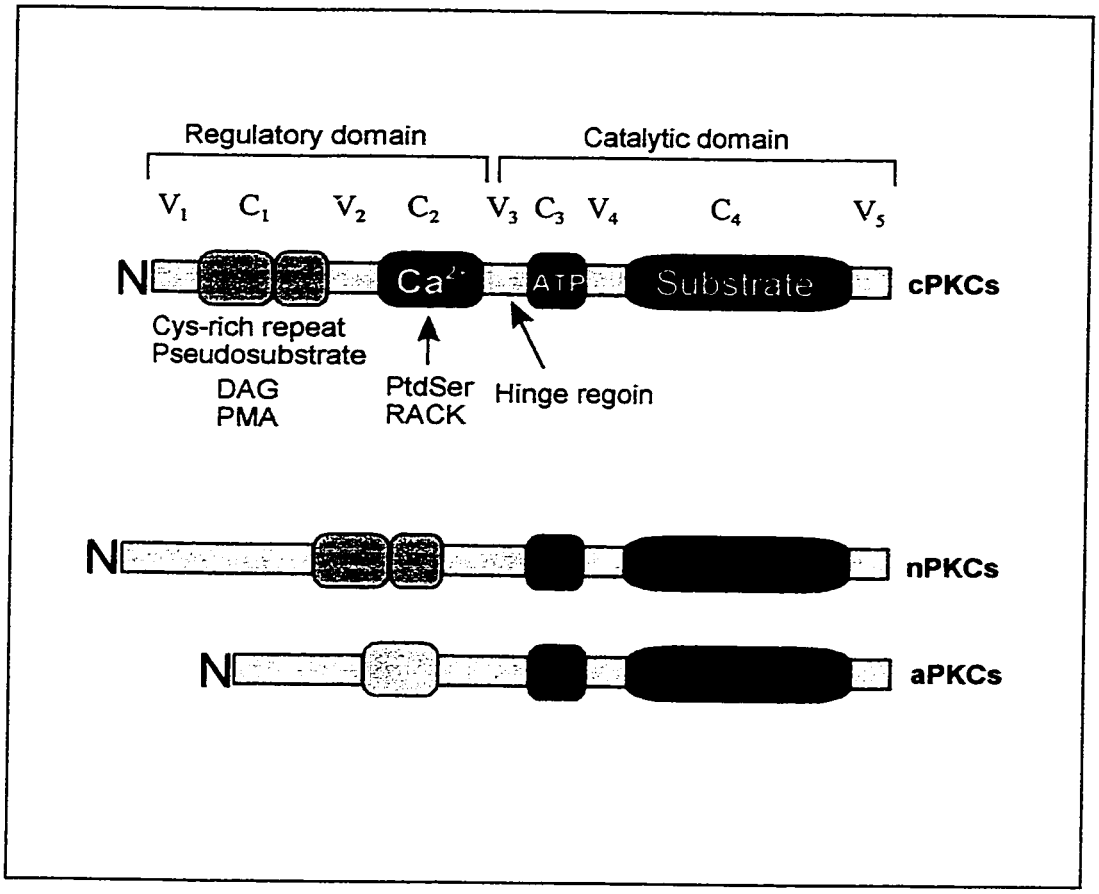

Fig 1.6. Structure of PKC isozymes. PKCs are classified on the basis of their structure and co-factor requirement. cPKCs-conventional PKCs, nPKCs-novel PKCs, aPKCs-atypical PKCs, PtdSer-phosphatidylserine and RACK-receptors for activated C kinase (modified from Liu and Heckman, 1998).

independent activity (Quest, 1996). The V_3 domain is known as the hinge region which separates the N-terminal regulatory domain from the C-terminal catalytic domain (Hug and Sarre, 1993). This region also contains the site for the proteolytic cleavage by trypsin or the Ca^{2+}-dependent neutral proteases calpain I and II (Fig 1.7). The C_3 region has the ATP binding motif $XGXGX_2GX_{16}KX$ which is found to be conserved in most protein kinases (Hug and Sarre, 1993). Finally, the C_4 region contains the substrate binding site and the phosphate transfer region (Quest, 1996). Protein kinase C family can be classified into three groups based on their structure and activation requirements (Nishisuka, 1995). First, classical or conventional PKCs (cPKCs) which includes PKCα, βI, βII and γ (Liu and Heckman, 1998). They require Ca^{2+}, phospholipid, free fatty acids and diacyl glycerol (DAG) or phorbol esters for their activation (Blobe et al, 1996). Second, novel PKCs (nPKCs) which includes PKCδ, ε, η, μ and θ (Liu and Heckman, 1998). This group lacks the second conservative motif (C_2) which contains the binding site for calcium. Therefore, this group is activated in a Ca^{2+}-independent manner (Liu and Heckman, 1998). Third, atypical PKCs (aPKCs) which includes PKCζ, ι, λ (Liu and Heckman, 1998). Members of this group are Ca^{2+}- and DAG-independent because they lack binding sites for these messengers (Fig 1.5) (Lui and Heckman, 1998).

Activation of PKC requires a combination of activators and cofactors which differ according to the group to which the isozyme belongs (Quest, 1996). In general,

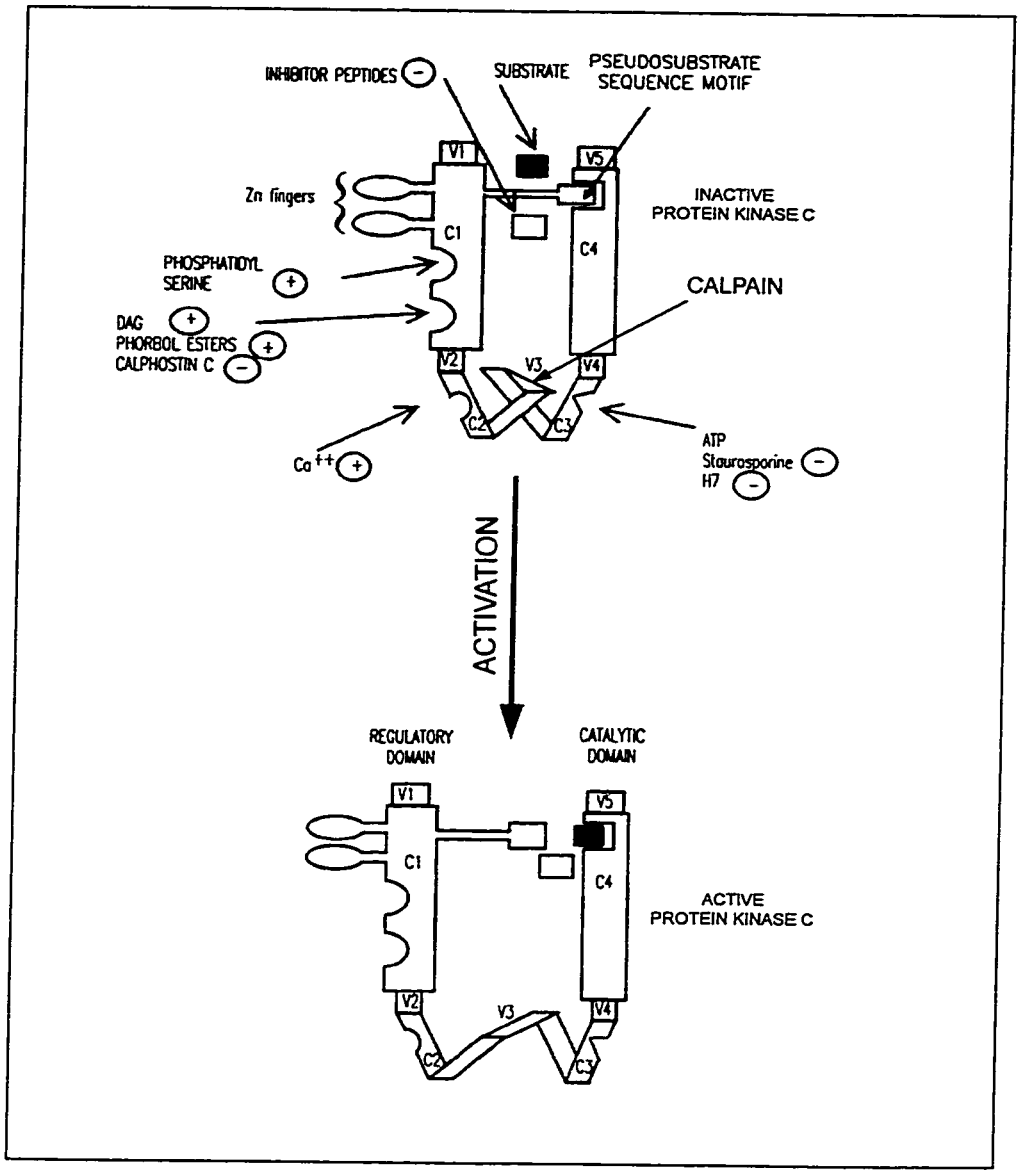

Fig 1.7. Proposed structure and mechanism of protein kinase C (PKC) activation. The four conserved structural motifs (C_1-C_4) and the five variable ones (V_1-V_5) are shown, together with the catalytic and regulatory parts of the enzyme. Site of binding of an inhibitory peptide is shown (modified from Azzi et al, 1992).

PKC needs diacylglycerol (DAG) and Ca^{2+} as well as phospholipids such as phosphatidylserine (PtdSer), phosphatidylcholine (PtdCho) and phosphatidylinositol (PtdIns) for activation (Fig 1.7) (Azzi, et al 1992). Free fatty acids are also requirements for activation of some PKC isozymes (Newton et al, 1 998). Phorbol esters, a tumor promoting agent, can replace DAG as a PKC activator (Crabos et al, 1991; Quest, 1996). However, phorbol esters and DAG differ in that phorbol esters appear to have more potent effect and to be more metabolically stable leading to more prolonged activation of PKC in cells and *in vitro* (Blobe et al, 1996). Activation of PKC is thought to involve its translocation from the cytosol to the plasma membrane (Newton et al, 1998). However, increasing evidence now exists to suggest that individual PKC isozymes can translocate to subcelluar locations including vesicles, nuclear structures and cytoskeletal compartments (Keenan and Kelleher, 1998). The subcellular localization of a specific isoform may directly control the potential of that isoform to perform distinct function (Quest, 1996). The targeting of PKCs to distinct subcellular compartments could restrict their access to potential substrates. On the other hand, receptors for activated PKC have been identified and named receptors for activated C kinase (RACK) (Monchly-Rosen et al, 1991). Binding of PKC to these receptors was saturatable and specific and was enhanced in the presence of DAG or phorbol esters (Azzi et al, 1992). RACK is thought to play an important role in translocation of activated PKC to specific cellular compartments.

A detailed exploration of the cellular regulation of individual PKC isozymes and their subcellular localization is a complex matter and lies beyond the scope of this introduction. However a series of recent excellent reviews on the subject are now available (Azzi et al, 1991; Dekker and Parker, 1994; Nishizuha, 1995; Blobe, 1996; Quest, 1996; Jaken, 1996; Majewski et al, 1997; Liu and Heckman, 1998; Mellor and Parker, 1998; Keenan and Kelleher, 1998; Vaughan et al, 1999).

2. Platelet Protein kinase C (PKC).

The state of platelet activation is precisely controlled by multiple excitatory and inhibitory extracellular stimuli. These stimuli are transformed into intracellular biological responses through a *s*eries of surface receptors present at the platelet plasma membrane. Platelet membrane receptors are usually coupled to intracellular enzymes whose activation results in generation of intracellular second messengers (Kroll and Sullivan, 1998). One of the major activation pathways in platelets is the hydrolysis of membrane phospholipids by G-protein activated phospholipase C (PLC) (Peerschke and López, 1998). This process leads to the production of two important second messengers, 1,4,5-triphosphoinositol (IP_3) and *sn*-1,2-diacylglycerol (DAG) (Peerschke and López, 1998). The main intracellular target of DAG is the activation of PKC supported by the elevation of the cytoplasmic free Ca^{2+} induced by IP_3 (Peerschke and López, 1998).

Platelets have been shown to contain at least nine different isoforms of PKC including PKCα, βI, βII, γ, δ, ε, ζ, η, and θ (Crabos et al, 1991; Carbos et al, 1992; Baldassare et al, 1992; Wang et al, 1993; Khan et al, 1993). Platelet PKC was found to translocate in response to stimulation of platelets with thrombin (Baldassare et al, 1992), platelet activating factor (PAF, Wang et al, 1993), oleic acid (Khan et al, 1993) or vasopressin (Crabos et al, 1992). PKC is also activated and translocated in response to the direct PKC activator phorbol ester (Crabos et al, 1991). Studies using direct PKC stimulation have revealed the involvement of PKC activation in platelet secretion, aggregation (without shape change) and metabolism of arachidonic acid (Kroll and Sullivan, 1998). However, the mechanism(s) by which PKC mediate these responses are not well understood. One of the major cellular events, as consequence of PKC activation, is the phosphorylation of a 47 KDa protein which in many publications has been used as a marker for platelet PKC activation (Kroll et al, 1993; Hashimoto et al, 1997; Freedman et al, 1996). The gene for this protein was later cloned, characterized and given the name pleckstrin (platelet C kinase substrate) (Imaoka et al, 1983; Tyers et al, 1988). Pleckstrin was found to be a major PKC substrate present in haematopoietic cells and absent from non-haematopoietic cells (Gailani et al, 1990). Several functions have been attributed to the protein including inositol triphosphate 5'-phosphatase (Connolly et al, 1986), lipocortin (Touqui et al, 1986), the α subunit of pyrovate dehydrogenase (Chiang et al, 1987) and an actin polymerization control protein (Hashimoto et al, 1997). Phosphorylation of pleckstrin

seems to correlate with platelet activation and it is thought to be involved in the PKC-dependent pathway of platelet dense-granule secretion (Walker and Watson, 1993; Sloan and Haslam, 1997; Coorssen et al, 1990; Rotondo et al, 1997), and aggregation (Brooks et al, 1990; Freedman et al, 1996). However, no direct relationship between pleckstrin phosphorylation and platelet secretion has been shown. All the studies that dealt with this issue were based on the observations that activation or inhibition of pleckstrin phosphorylation using non-specific activators or inhibitors of PKC were always accompanied by an increase or a decrease in platelet secretion (Yamada et al, 1987; Walker and Watson, 1993; Sloan and Haslam, 1997). On the other hand, there is indirect evidence which suggests that phosphorylated pleckstrin inhibits the accessibility of polyphosphoinositide to hydrolysis by PLCβ and PLCγ (Abrams et al, 1995). Phosphopleckstrin was also shown to inhibit platelet phosphatidylinositol-4,5,bisphosphate 3-kinase (Abrams et al, 1996).

Another platelet protein which becomes phosphorylated upon activation of PKC is the 20 KDa myosin light chain (MLC) (Ingaki et al, 1984). This protein was also implicated in platelet secretion (Inagaki et al, 1984) and aggregation (Takano, 1994). Myosin comprises approximately 12% of the total platelet protein (Seiss, 1989). It is composed of one pair of heavy chains (200 KDa) and two pairs of light chains (20 and 15 KDa) (Seiss, 1989). Phosphorylation of MLC is achieved by activation of a substrate specific Ca^{2+}-calmodulin dependent kinase, the myosin light chain kinase (MLCK) (Seiss, 1989). However, phorbol esters and bioactive

diacylglycerol, when added to intact platelets also evoke PKC-dependent phosphorylation of myosin light chain at sites different from those phosphorylated by MLCK. In stimulated platelets, the rate and extent of myosin phosphorylation parellel the association of myosin with the platelet cytoskeleton (Seiss, 1989). Phorbol ester activation of platelets has been shown to increase phosphorylation of other unidentified phosphoproteins whose role(s) is yet unknown (Patel et al, 1994; Turini et al, 1993).

PART TWO:

Role of the cytoskeleton in exocytosis and its regulation by PKC.

A great deal of information about the role of the F-actin cytoskeleton in secretion has been obtained utilizing adrenal chromaffin cells as a model (Trifaró et al, 1984; Trifaró et al, 1989; Trifaró et al, 1998a). It was observed that chromaffin cells have a submembranous region (~150 nm) that is almost devoid of secretory vesicles in resting cells (Vitale et al, 1995). Fluorescence microscopy using actin antibodies (Lee and Trifaró, 1981), and rhodamine-phalloidine (Lee and Trifaró, 1981; Cheek and Burgoyne, 1996; Vitale et al, 1991), a probe for filamentous actin (Faulstisch et al, 1988), revealed the presence of a cortical subplasmalemal network of actin filaments in chromaffin cells (Lee and Trifaró, 1981; Cheek and Burgoyne, 1996). This cortical actin network serves as a barrier preventing secretory vesicles from reaching and interacting with the plasma membrane (Trifaró et al, 1998a; Trifaró

et al, 1998b).

Fluorescence microscopy has demonstrated that catecholamine secretion from chromaffin cells is accompanied by a focal and transient disruption of the cortical F-actin network (Cheek and Burgoyne, 1996; Trifaró et al, 1989; Marxen and Bigalke, 1991; Vitale et al, 1991; Nakata and Hirokawa, 1992). A decrease in F-actin and concomitant increase in G-actin, as evaluated by the DNase inhibition assay (Cheek and Burgoyne, 1986; Trifaró et al, 1989), and a reduction in the amount of actin associated with the cytoskeleton also occurs upon chromaffin cell receptor stimulation or membrane depolarization (Burgoyne et al, 1989; Wu et al, 1992). Moreover, in intact or permeabilized cells, the presence of substances that destabilize F-actin, such as cytochalasin D or DNase I, enhances stimulation-induced catecholamine secretion (Friedman et al, 1980; Lelkes et al, 1986; Sontag et al, 1988). The above observations suggest that the cortical actin network must be locally removed for secretion to occur. Such cortical F-actin network has not been demonstrated in platelets. However, recent experimental data have suggested that actin disassembly, perhaps at certain specific areas, mediate the platelet release reaction (Marcu et al, 1996; Marcu et al, 1998; Elzagallaai et al, 1998; Elzagallaai et al, 2000).

The idea that PKC may be involved in the regulation of the cytoskeleton came from a number of observations obtained from experiments performed on several cell types. These observations include: 1)PKC activators such as phobol esters cause cytoskeletal rearrangement that could be demonstrated using immunocytochemical

techniques (Vitale et al, 1992), 2) colocalization of some PKC isoforms with cytoskeletal proteins (Vaughan et al, 1999), 3) translocation of some PKC isoforms to the cytoskeleton compartment upon activation (Quest, 1996), 4) Some cytoskeletal proteins are PKC substrates (Ohta et al, 1987; Naka et al, 1983), and 5) specific PKC inhibitors block agonist-induced cytoskeletal rearrangement (Vitale et al, 1992). In chromaffin cells, activation of PKC by phorbol esters induces cortical F-actin disassembly and increased the initial rate of exocytosis in response to nicotine stimulation (Vitale et al, 1995). This effect was blocked by specific PKC inhibitors such as staurosporine and calphostine C (Vitale et al, 1992). In addition, using the patch clamp technique which allows for the direct measurement of the increase in membrane capacitance as an index of exocytosis, Vitale et al (1995) have described that PKC activation enhances secretion from chromaffin cells by increasing the size of the readily releaseable pool of secretory vesicles. Furthermore, reorganization of F-actin network during PKC-induced exocytosis has also been shown to occur in other cell types (Goodall et al, 1997; Danks et al, 1999).

The Myristoylated Alanine Rich C Kinase Substrate (MARCKS).

While the importance of protein kinse C in the regulation of F-actin cytoskeleton and mediation of exocytosis is well established, the overall picture of how PKC is involved in this interplay is not fully clear. As mentioned earlier, many cytoskeletal proteins are PKC substrates including actin (Ohta et al, 1987) and myosin

light chain (Naka et al, 1983). However, MARCKS, another substrate of PKC, has recently attracted the attention as the link between PKC activation and rearrangement of F-actin cytoskeleton (Hartwig et al, 1992; Underhill and Aderem, 1997; Goodall et al, 1997).

In 1982, Wu and co-workers described the existence of an acidic protein with apparent molecular weight of approximately 87 KDa in isolated rat brain synaptosomes. The protein, named Mr 87K, was found to become phosphorylated in response to depolarization-induced Ca^{2+}-influx and when cytosolic extracts from rat brain were treated with Ca^{2+} and phospholipids, suggesting that this protein was a substrate of a Ca^{2+}/phospholipid-dependent protein kinase (Wu et al, 1982). This protein was shown to be dissimilar to protein I, a phosphoprotein present in synaptosomes which migrates at the same molecular weight (Wu et al, 1982). The two proteins have different subcellular distributions, isoelectric points, peptide maps and effectiveness as substrates for various classes of protein kinases (Wu et al, 1982). Subsequently, in 1983, Rozengurt and colleagues provided the first evidence that this protein was a substrate of protein kinase C by demonstrating that downregulation of PKC in Swiss 3T3 fibroblasts led to a loss in the ability of phorbol esters to induce phosphorylation of Mr 80K, a protein equivalent to the synaptosomal Mr 87K. Furthermore, this group have also shown that agents that do not activate the phosphoinositol pathway (e.g., insulin) were unable to induce phosphorylation of Mr 80 K protein (Rodriguez-Pena and Rozengurt, 1986). The primary sequences of this

protein originated from bovine (Stumpo et al, 1989), chicken (Graff et al, 1989), mouse (Seykora et al, 1991; Brooks et al, 1991), rat (Erusalimsky et al, 1991) and human (Harlan et al, 1991; Sakai et al, 1992), have been published. These cloned proteins have appeared to be species variants of the same protein (Blackshear et al, 1992). Being heavily myristoylated and rich in alanine residues, this protein kinase C substrate was later named myristoylated alanine rich C kinase substrate or MARCKS (Aderem, 1992; Blackshear, 1993).

MARCKS is an acidic (pI 4.1-4.4) and heat-stable protein that is unusually rich in alanine, glycine, proline and glutamic acid (Underhill and Aderem, 1997). The predicted molecular weight based on the amino acid sequence ranges from 31,891 daltons (bovine) to 27,732 daltons (chicken). However, the protein migrates on an SDS-PAGE at an apparent size of Mr 60,000 (chicken) to Mr 87,000 (bovine) (Blackshear, 1993). This discrepancy is thought to be due to the rigid rod shape of the protein and to its very acidic nature which affect its migration during electrophoresis (Aderem, 1992). MARCKS consists of three distinct domains: 1) a heavily myristoylated N-terminus which mediates the binding of the protein to membranes; 2) a highly conserved MH2 domain of unknown function; and 3) a basic effector domain (phosphorylation site domain, PSD) containing the PKC phosphorylatable serine residues, and Ca^{2+}/calmodulin and actin binding sites (Fig 1.8, Aderem, 1992). The purified protein appears under electronmicroscopy as rods having dimensions of 3.7-5.1 nm × 35.6 ± 2.8 nm (Hartwig et al, 1992). Physical measurements indicate

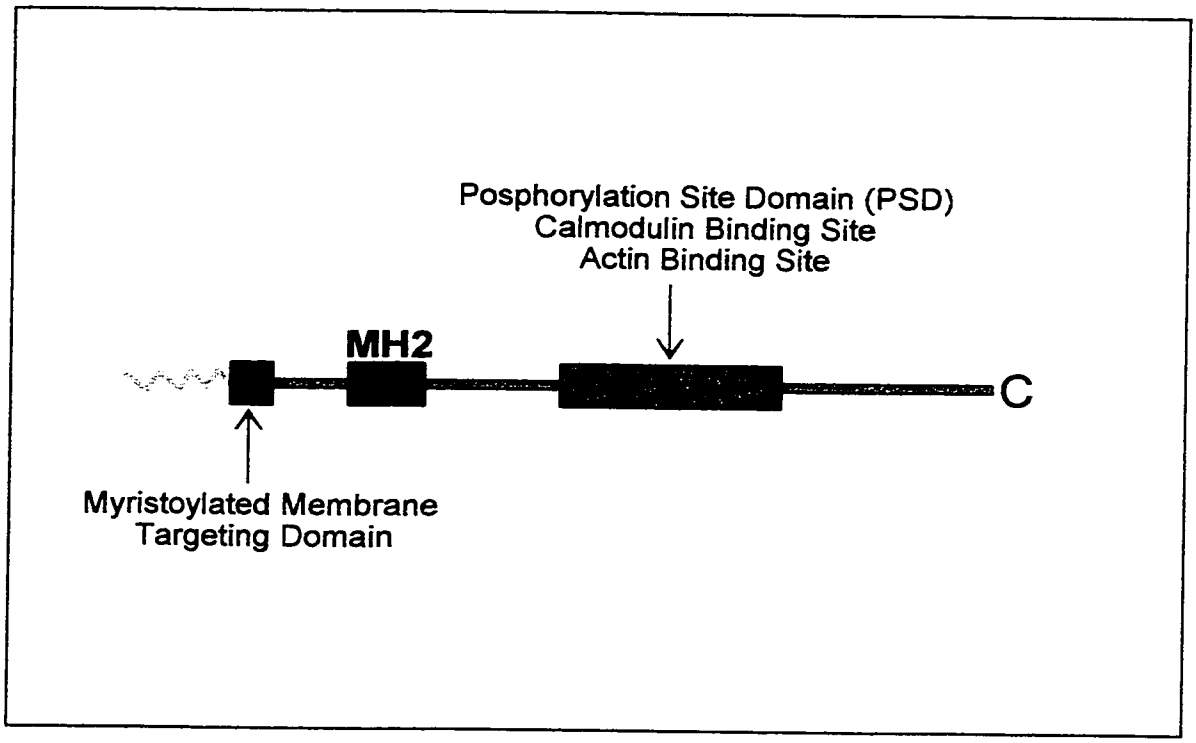

Fig 1.8. Schematic structure of the MARCKS molecule.

that the protein is composed nearly entirely of a random coil with small α-helical content (Underhill and Aderem, 1997). One of the most interesting aspects of MARCKS' cellular profile is its reciprocal interaction with other proteins. MARCKS binds to calmodulin in presence of Ca^{2+} and phosphorylation of MARCKS by PKC prevent this binding (Hartwig et al, 1992). MARCKS also binds to the sides of actin filaments and cross-links them (Hartwig et al, 1992). The phosphorylation of MARCKS by PKC also abrogates its actin cross-linking activity as does its binding to the Ca^{2+}/calmodulin complex (Aderem, 1992). Another important property MARCKS is that it binds to cellular membranes (Taniguchi et al, 1993; Kim et al, 1994; Vergéres et al, 1995; Seykora et al, 1996; Arbuzova et al, 1998). This binding is mediated through two structural domains present in MARCKS: the myristoylated N-terminus and the highly basic PSD domain (Aderem, 1992; Blackshear, 1993). The fatty acid of the myristoyl moiety inserts into the hydrophobic interior of the bilayer membrane while the basic domain interacts with the membrane acidic phospholipids (Aderem, 1992). Phosphorylation of MARCKS by PKC results in introduction of negatively charged phosphate groups into the basic domain changing its net charge from +13 to +7 and reversing its interaction with the membrane (Thelen et al, 1991; Underhill and Aderem, 1997). The process can happen also in a reversed manner as dephosphorylation of the protein lead to its reassociation with the membrane compartment (Seykora et al, 1996). This versatile mechanism is called "the myristoyl electrostatic switch" and seems to be very important for the cellular function of

MARCKS (Thelen et al, 1991; McLaughlin and Aderem, 1995).

MARCKS is an F-actin binding and cross-linking protein (Hartwig et al, 1992). It has been implicated in many cellular functions including cell motility, phagocytosis and exocytosis, all of which require reorganization of the F-actin cytoskeleton (Goodall et al, 1997; Underhill and Aderem, 1997). MARCKS co-localize with vinculin, talin and PKCα in podosomes, the macromolcular complexes which mediate adhesion of macrophage to the substratum (Rosen et al, 1990; Underhill and Aderem 1997). Activation of PKC by phorbol esters results in displacement of MARCKS but not vinculin or talin, and this translocation of MARCKS is associated with macrophage spreading (Rosen et al, 1990). Similarly, agonist-induced rearrangement of cytoskeleton in cultured endothelial cells is accompanied by phosphorylation of MARCKS (Zhao et al, 1998). MARCKS also is involved in macrophage phagocytosis and maturation of phagosomes (Allen and Aderem, 1995). Over-expression of MARCKS in OCM-1 cells, which are tumor derived choroidal melanoma cells, has led to enhancement of cell spreading and formation of membrane processes (Manenti et al, 1997). At the same time, there was an increase in tyrosine phosphorylation of paxillin and its co-localization with vinculin suggesting a role of MARCKS in the reorganization of cytoskeleton during focal contact formation (Manenti et al, 1997). Using subcellular fractionation and immunocytochemical staining, Goodall et al (1997) had demostrated that upon phorbol ester treatment, PKCα, but not PKCε or PKCζ, was translocated from the

cytosol to the membrane compartment in an HS-SY5Y neuroblastoma cell line. In the same preparation, MARCKS was found to become phosphorylated and to translocate from the membrane to the cytosol upon phorbol ester treatment (Goodall et al, 1997). These effects were closely correlated with the enhancement by phorbol esters of carbachol-induced noradrenaline (NA) release. Furthermore, the phosphorylation and the translocation of MARCKS were found to be blocked by PKC inhibitors as was the enhancement by phorbol ester of NA release (Goodall et al, 1997). These results suggest the involvement of some PKC isozymes and MARCKS in exocytosis.

The hypothesis for a role of MARCKS in organization of cytoskeleton and regulation of exocytosis is that in resting cells, MARCKS, act as F-actin cross-linker, thus increasing the mechanical strength of the F-actin network and, as a membrane bound anchor, links actin filaments to the cytoplasmic face of plasma membrane (Vitale et al, 1995; Underhill and Aderem, 1997). Activation of PKC pathway will result in phosphorylation of MARCKS (Rodríguez-Pena and Rozengurt, 1986). This will decrease its F-actin cross-linking activity and displace the protein from the membrane leading to disruption of the cortical actin network and allow secretory vesicles to reach and interact with the plasma membrane as a prelude to exocytosis (Vitale et al, 1995; Goodall et al, 1997). This highly regulated and time limited process is followed by subsequent dephosphorylation of MARCKS then its reassociation with the membrane and regaining of its F-actin cross-linking activity (Hartwig, et al 1992). The versatile regulation of MARCKS and its ability to bind

many cellular components is indicative of the delicate role of MARCKS in many cell functions. However, other closely related proteins such as MacMARCKS, also named MARCKS related protein (MRP) and F52 (Underhill and Aderem, 1997), seem to have a similar structure and cellular profiles and may complement or even replace MARCKS in function. Nevertheless, MARCKS deficiency in mice is lethal and leads to abnormal neuronal development suggesting a role of MARCKS in CNS maturation (Stumpo et al, 1995). Except for some studies in neuronal cells, fibroblasts and macrophages, our knowledge of the function and regulation of MARCKS is extremely poor and is in need of further research.

PART THREE:

Platelet permeabilization techniques.

The plasma membrane primary function is to maintain the integrity of the cell and its internal components but it also represents the main obstacle for cell biologists to freely access the cell interior. Therefore, attempts have been made to overcome this barrier using several techniques started with mechanical techniques for skinning muscle fibers followed later by the use of physical and chemical agents to selectively produce lesions of plasma membranes (Schulz, 1990). Cell permeabilization is a method used to non-selectively increases the permeability of the cell membrane without disrupting other cell components. Successful cell permeabilization should not

compromise the functional or structural integrity of the cell, or cause massive leakage of cell components and/or organelles into the medium. Permeabilized cells should, then, maintain their normal responsiveness to various stimuli. Using permeable cell models (leaky cells) for biological experiments has an advantage over using intact cells in that, permeable cells enable us to perform a controlled manipulation of the cell interior. It also provides us with more accurate information about intracellular biological processes than cell-free systems do.

Techniques used for cell permeabilization include: electropermeabilization (Knight and Scrutton, 1993), treatment with mild non-ionic detergents (e.g. digitonin or saponin) (Lineberger et al, 1989), and pore-forming bacterial toxins (e.g., α-toxin and streptolysins) (Buckingham and Duncan, 1983). Other permeabilization techniques include osmotic shock, brief freeze/thaw, treatment with Sendai virus, ATP, and the use of Ca^{2+}-chelators (e.g., EDTA and EGTA) (Schulz, 1990).

A. Electropermeabilization.

Electropermeabilization is also known as "electroporation," "electropermeation," or "electrical breakdown" (Schulz, 1990). The principle of this technique is that when suspended cells are exposed to an electric field, a potential develops across their membranes (Knight and Scrutton, 1993). The maximal potential will develop at two points on the membrane in line with the applied electrical field

(Knight and Scrutton, 1993). This will lead to creation of two small pores in the membrane and by repeating the process more pores will be formed (Knight and Scrutton, 1993). This procedure is achievable by placing suspended cells, such as platelets, between two electrodes and subjecting them to a high intensity voltage (kV/cm range) for short period of time (nsec to μsec range) (Knight and Scrutton, 1993). The main shortcoming of this technique is that the created pores are not permanent as the membrane tends to reseal itself shortly after treatment (Hersey and Pérez, 1990). On the other hand, the size of the localized damage to the membrane is quite small (table 1.1) (Schulz, 1990). In addition, electropermeabilization seems to have some effect on platelet morphology as they increase in volume during the process (Knight and Scrutton, 1993).

B. Pore-forming bacterial toxins.

These toxins include α-toxin from streptococcus aureus and streptolysins from β-hemolytic streptococci (Schulz, 1990; Buckingham and Duncan, 1998). Alpha-toxin creates transmembrane pores in the plasma membrane (Schulz, 1990). The toxin assembles into a characteristic ring structure in the target membrane (Schulz, 1990). It becomes closely associated with the lipid bilayer and create stable transmembrane pores with a diameter of 2-3 nm, allowing permeation of smaller molecules up to 1,000 daltons such as ions and nucleotides (Schuliz, 1990). Streptolysins

Method	Pore diameter (nm)	Permeant molecule	(KDa)
Elctroporation	~2	Ions, nucleotides	Up to 1
Digitonin	8-10	Enzymes	Up to 200
Saponin	8-10	Enzymes	Up to 200
α-toxin	2-3	Ions, nucleotides	Up to 1
Streptolysin-O	>15	Enzymes, immunoglobulins	>200

Table 1.1. Pore size and molecular masses of permeant solutes in cells permeabilized by different methods. (Modified from Schulz, 1990).

(streptolysin-O and streptolysin-S) create pores heterogeneous in size and structure. Streptolysin-S lyses cells by a colloid-osmotic process while streptolysin-O is thought to produce larger primary lesions, resulting in noncolloid-osmotic lysis (Buckingham and Duncan, 1998). Treatment of platelets with streptolysin-O caused 100% leakage of large cytosolic proteins such as PKC and pleckstrin within 10 minutes of permeabilization (Sloan and Haslam, 1997).

C. Non-ionic detergent permeabilization.

Selective permeabilization of the plasma membrane can be achieved by treating the cells with mild non-ionic detergents such as the plant glycosides digitonin and saponin. Digitonin is a steroid glycoside detergent which specifically interacts with and sequesters cholesterol and other β-hydroxysteroids from the plasma membrane (Schulz, 1990). The removal of these lipids from the plasma membrane leads to creation of pores of sufficient size to permit entry of molecules as large as immunoglobulins (Lineberger et al, 1989) The selectivity of digitonin-induced permeabilization of plasma membranes is thought to be due to the high molar ratio of cholesterol to phopholipids in the plasma membrane compared to other intracellular membranes, or may be attributed to the reduced accessibility of the detergent to intracellular membranes (Fiskum et al, 1980). Even at digitonin concentrations as

high as 3000 μg/ml neither lysosomes nor mitochondria release their soluble enzymatic content (Schulz, 1990). Saponin, however, is less selective and has been used to permeabilize the membranes of the endoplasmic reticulum (Schulz, 1990). This specificity of digitonin provides a major advantage over the use of other permeabilizing agents such as triton X-100 and toluene, which work by non-selective removal of membrane lipids (Schulz, 1990). The degree of damage that occurs to the membrane depends on the concentration of digitonin and the time of incubation. Studies of the effect of digitonin on the electrical conductance through artificial membrane have shown that it induced channel-like fluctuations in plannar bilayers consisting of either DphPC (diphytanoylphosphatidylcholine) or DphPC and cholesterol (2/1, w/w) (Schulz, 1990). The amounts of detergent required to increase the conductance of cholesterol-free and cholesterol-rich membranes were about 0.2 mg/ml and 10 μg/ml, respectively (Schulz, 1990). It is thought that the lipophilic heads of digitonin molecules interact with cholesterol and form a ring with a central hydrophilic hole in a micellar arrangement (Schulz ,1990).

Among other platelet permeabilization procedures digitonin-permeabilization is the least frequently used technique. However, good results have been obtained using this detergent with washed human platelets (Lineberger, 1989; Marcu et al 1996; Elzagallaai et al, 2000; this thesis).

PART FOUR:

Statement of the problem.

The mechanism of platelet secretion has always been a subject of controversy. After the concept of "platelet release reaction" was largely accepted (Sherry, 1976; Hartwig, 1998), a plethora of experimental data has been gathered to strongly suggest that platelet secretion mechanism would be no different from that of any other secretory cell (Ginsberg et al, 1980; Carroll et al, 1982; Polasek et al, 1987; Zucker-Franklin et al, 1995; Marcu et al, 1996; Marcu et al, 1998; Elzagallaai et al, 1998; Elzagallaai et al, 2000). This highly conserved mechanism is utilized even by yeast (Finger and Novick 1998) and proved to be an energy efficient mechanism that evolution has kept in used even in neurons (Trifaró et al, 1989). Furthermore, the concept of "platelet release reaction" does not explain a role for the Ca^{2+}-activated filamentous actin severing proteins such as scinderin in platelet secretion. Recombinant scinderin was found to enhance Ca^{2+}-induced serotonin release from permeabilized platelets (Marcu et al, 1996; Marcu et al, 1998). In addition, in another secretory system, the chromaffin cell, a PKC activator such as PMA was found to induce cortical F-actin disassembly and increase noradrenaline secretion in response to stimulation (Vitale, 1995). In this regard, PMA also induces serotonin release from platelets (Coorssen and Haslam, 1993) and PKC inhibition or downregulation blocked agonist-induced platelet secretion (Patel et al, 1994). The aforementioned evidence

suggests that a PKC pathway mediates, at least in part, platelet exocytosis. In a search for clues to understand the events involved in platelet secretion, and in particular, the role of PKC in this process, we have asked a series of questions and have designed experiments to address the following:

1. To develop and characterize a permeabilized platelet preparation and use it to address the role of PKC and its substrates in platelet secretion.
2. To investigate whether MARCKS is present in platelets and, if this is the case, study the role of this protein in platelet exocytosis.
3. If MARCKS is found to be present in platelets, the possibility that MARCKS phosphorylation mediates PKC-induced platelet secretion will be studied. Efforts will be made to correlate the degree of MARCKS phosphorylation to PKC effects on serotonin release.
4. The effect of thrombin on serotonin release and MARCKS phosphorylation will be studied.
5. Peptides corresponding to the phosphorylation site domain of MARCKS (MPSD) will be designed and used in serotonin release and phosphorylation studies on permeabilized platelets stimulated by PMA and thrombin.
6. A comparison of responses obtained by direct stimulation of PKC by PMA with those responses obtained through the activation of the enzyme by stimulation of platelet by thrombin will indicate the contribution to the release reaction of the PKC-MARCKS transduction pathway.

Chapter (II)

Materials and Methods

A. *Materials.*

Peptides that correspond to the phosphorylation site domain of MARCKS (MPSD, KKKKKRFSFKKSFKLSGFSFKKNKK), and to the same domain but with the serine residues replaced by alanine residues, (Ala-MPSD, KKKKKRFAFKKAFKLAGFAFKKNKK), were custom-made by Research Genetics, Huntsville, AL, USA. PMA (Phorbol 12-Myristate 13-Acetate), digitonin and adenosine 5'-triphosphate (ATP, disodium salt) were obtained from Sigma, Oakville, ON, Canada. PMA was prepared as a stock solution in DMSO, and stored at –20 °C. Thrombin was purchased from Chrono-log inc.,Hovertown, PA, USA. Thrombin was dissolved in phosphate-buffered saline (PBS: 130 mM NaCl, 100 mM Na-phosphate, pH 7.2) and stored at -20 °C until further use. [^{3}H]serotonin (5-HT) was purchased from DuPont, Boston, MA, USA. Carrier-free [^{32}P]-orthophosphate was from Amersham, Oakville, ON, Canada. All chemical and solvents were of analytical grade. Antibodies were obtained from the following sources: a) mouse monoclonal IgG raised against the C-terminal domain of human MARCKS and rabbit polyclonal IgG raised against a synthetic peptide corresponding to amino acids 641-673 of C-terminus of PKC (recognizes α, β and γ isoforms of PKC at 82 KDa) (Upstate, Lake Placid, NY, USA); b) goat polyclonal raised against a peptide mapping at the C-terminus of human MARCKS and goat polyclonal raised against a peptide mapping

at the N-terminus of human MARCKS (Santa Cruz Biotechnology, Santa Cruz, CA, USA); c) mouse monoclonal raised against the N-terminus of human pleckstrin (Tranduction Laboratories, Lexington, KY, USA); d) HRP-conjugated goat antimouse IgG (BioRad, Mississauga, ON, Canada); e) HRP conjugated affinity purified $F(ab')_2$ fragment rabbit antigoat $F(ab')_2$ fragment specific; f) HRP conjugated goat antirabbit IgG; g) HRP conjugated $F(ab')_2$ fragment donkey antimouse IgG; h) CY^3 conjugated affinity purified $F(ab')_2$ fragment donkey antimouse IgG. (e to h from Jackson Immuno Research Laboratories Inc., West Grove, PA, USA); i) mouse monoclonal IgG raised against human CD41a (PharMingen, Mississauga, ON, Canada). Rhodamine-phalloidin was purchased from Molecular Probes, Eugene, Oregon, USA. Rhodamine-pholloidin was dissolved in methanol and stored at -20°C until use.

B. Methods.

1. Source of Platelets.

Platelet-rich plasma was obtained from the blood bank of Ottawa Red Cross, Ottawa, ON, Canada. The viability of platelets was tested by measuring their aggregation as response to stimulation with 1U thrombin/ml in a whole blood aggregometer (see platelet aggregation section). Only platelet preparations which have typical responses to thrombin stimulation were used. Platelet-rich plasma was centrifuged at 200 g for 15 min to eliminate red blood cells. The supernatant thus

obtained was centrifuged at 800g for 15 min to obtain a platelet sediment.

2. Platelet Permeabilization.

Platelet permeabilization is a technique used to introduce into the platelet cytosol substances to which intact platelets are impermeable. One such technique is the use of a non-ionic detergent (i. e. digitonin) permeabilization method. The principle of this technique is explained in some detail in the introduction to this thesis. The permeabilized platelet preparation used in these studies has been first characterized in our laboratory (see Results).

(i) Permeabilization.

The platelet pellet was resuspended in Ca^{2+}-free Locke's solution (mM: NaCl, 154; KCl, 2.6; K_2HPO_4, 2.14; KH_2PO_4, 0.85; $MgCl_2$, 1.2; glucose, 10; and EGTA, 2.0; pH 7.2). After a wash with Ca^{2+}-free Locke's solution, the platelet concentration was adjusted to 7.5×10^8 platelet/ml in Ca^{2+}-free Locke's solution. Platelet aliquots were pelleted down by centrifugation and resuspended in K^+-glutamate buffer (mM: $MgCl_2$, 12.5; K^+-glutamate, 160; EGTA, 2.5; EDTA, 2.5; adenosine triphosphate (ATP), 5; HEPES, 20; pH 7.4,) (Kamiguti et. al., 1997) containing 15 μM digitonin and incubated at room temperature for 5 min. At the end of the permeabilization period, platelets were centrifuged at 1600 g for 20 s and permeabilized platelets were then resuspended in K^+-glutamate buffer to carry out the experiments.

(ii) Evaluation of platelet permeabilization.

The degree of permeabilization was determined using rhodamine-phalloidin (a probe for filamentous actin). Platelets are impermeable to this substance and only permeabilized platelets are stained positively. Intact platelets, and platelets permeabilized for different periods of time, were centrifuged at 1000 g for 10 min onto poly-L-lysine-coated glass slides (Electron Microscopy Sciences, Washington, PA, USA) using a bench top cytospin centrifuge (cytofuge 2. Stat Spin Inc., Norwood, Mass., USA). Platelets were then fixed in 3.7% formaldehyde for 20 min and stained with rhodamine-phalloidin (1:200 dilution) for 15 min at room temperature, washed three times with phosphate-buffered saline (PBS) and mounted in 50% glycerol/PBS. Platelet preparations were examined using incident fluorescent light microscope, pictures were taken and images were processed as described under Fluorescence Microscopy. The percentage of rhodamine phalloidin positive cells (permeabilized platelets) was then determined from the prints.

3. Platelet aggregation.

Platelet aggregation was measured using Whole Blood Dual Channel Lumi-aggregometer (Chrono-log inc.,Hovertown, PA, USA). The dual channel optical aggregometer is a fixed wavelength spectrophotometer with a sample chamber (or chambers) heated to 37°C. Provision was made for stirring of the sample because platelet to platelet contact is necessary for determination of *in-vitro* platelet

aggregation. The Chrono-log sample chambers are designed so that a beam of infra-red light shines through two cuvettes simultaneously, one containing the sample and the other containing the reference (the incubation buffer). Silicon photodiodes detect light that is able to pass through the sample. The sample is arbitrarily considered to be 0% light transmission or 0% aggregation, whereas the reference is considered to correspond to 100% light transmission or 100% aggregation. The difference in light transmission outputs between the photodiodes is transferred to a recording device (Fig. 2-1). Aliquots (0.5 ml) of either intact platelets suspended in either Ca^{2+}-free Locke's solution or regular Locke's solution (mM: NaCl, 154; KCl, 2.6; K_2HPO_4, 2.14; KH_2PO_4, 0.85; $MgCl_2$, 1.2; glucose, 10; and $CaCl_2$, 2.2; pH 7.2) or permeabilized platelet suspended in K^+-glutamate buffer were placed in siliconized glass cuvette positioned in the sample chamber and kept at 37°C under constant stirring using siliconized stirring bars. A reference sample (0.5 ml) of the buffer used was placed in similar cuvette positioned in the reference chamber. Following the addition of different substances (i.e. PMA or thrombin) platelet aggregation was monitored by recording the increase in light transmission through the sample cuvette using Chrono-log chart recorder model number 707.

4. Labelling of Serotonin Stores.

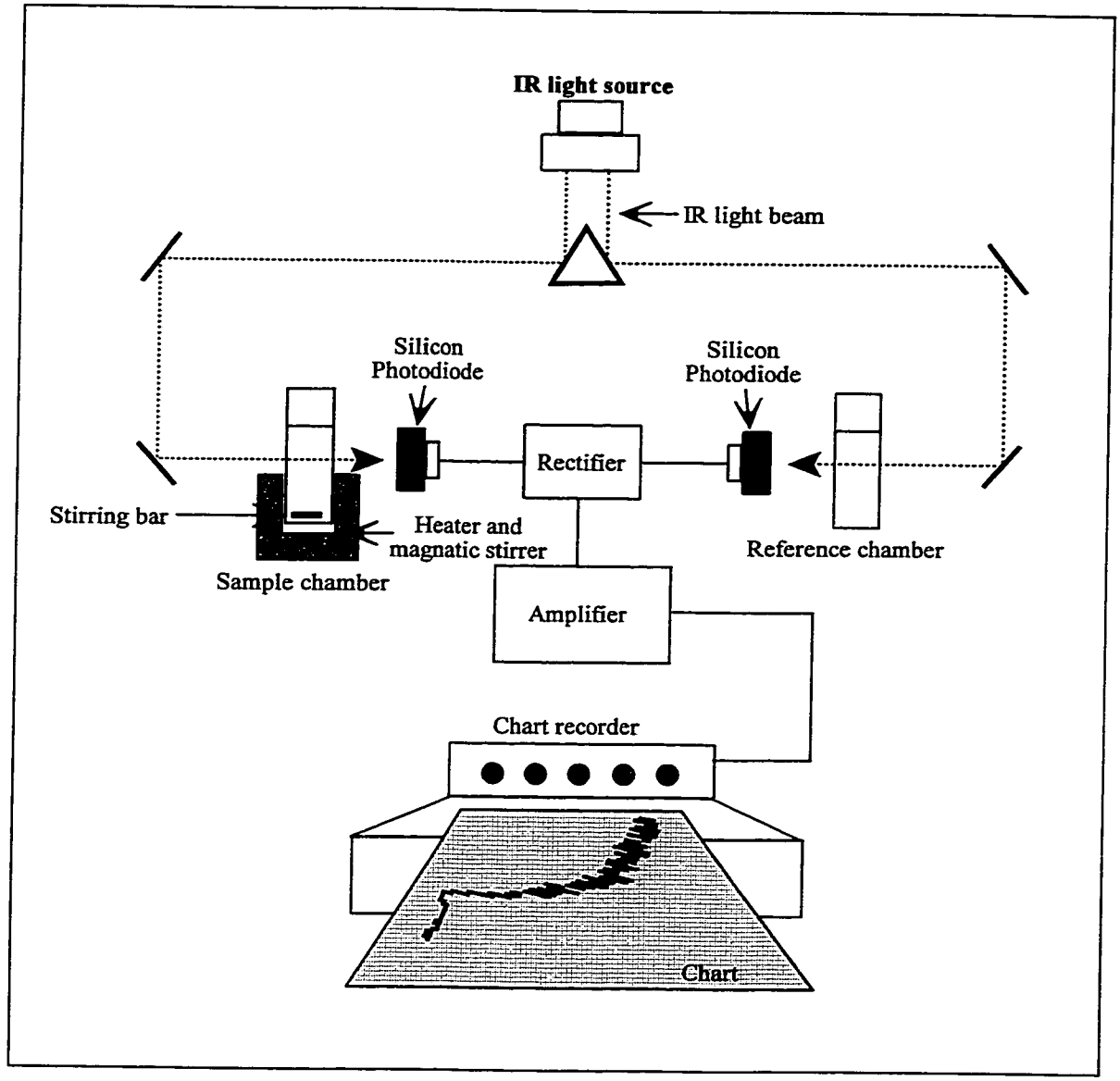

Fig 2.1. Schematic representation of the dual channel aggregometer.

The platelet pellet was resuspended in Ca^{2+}-free Locke's solution. After a wash with Ca^{2+}-free Locke's solution, the platelet concentration was adjusted to 7.5×10^8 platelet/ml. Platelets were then incubated at 37°C for 90 min with 0.6 nmol [^{3}H]5-HT/ml (specific activity = 25.4 Ci/mmol; DuPont, Boston, MA). After incubation, the [^{3}H]5-HT-labelled platelets were washed by incubation with six changes of Ca^{2+}-free Locke's solution over a 60-minute period before the experiments were commenced

5. Serotonin Release Studies.

Samples (100 µl) containing 7.5×10^8 platelets/ml of either permeabilized platelets suspended in K^+-glutamate buffer or intact platelets suspended in Ca^{2+}-free Locke's solution were incubated in the absence or presence of different secretagogues for the indicated period of time. Release experiments were terminated by addition of an equal volume of 6% glutaraldehyde in 0.1 M phosphate buffer (pH 7.4). Preparations were centrifuged at 1600 g for 1 min. Sediments were extracted with 200 µl of 10% trichloroacetic acid (TCA) and radioactivity in supernatants and TCA extracts was measured in a liquid scintillation spectrometer (Beckman Instruments, Fullerton, CA). Total [^{3}H]5-HT platelet content was determined by adding the radioactivity present in the incubation medium to that in the TCA extract. [^{3}H]5-HT output was expressed as a percentage of platelet total content, after subtraction of

values for spontaneous release. Values represent mean ± SEM.

6. [^{32}P]Pi Labelling of Platelets.

The platelet endogenous ATP pool was labelled according to the following procedure: Platelets (7.5 x 10^8/ml) suspended in Ca^{2+}-free Locke's solution were centrifuged at 800g for 2 min. The platelet sediment was resuspended in a phosphate free solution (buffer P) of the following composition (mM: NaCl, 145; KCl, 5; $MgSO_4$, 1; glucose, 10; HEPES, 25; EGTA, 0.5; pH 7.3) to give a platelet concentration of 5 x 10^8 platelet/ml. Platelets were incubated for 60 min in buffer P containing 150 μCi carrier-free [^{32}P]Pi/ml (Amersham, Oakville, ON, Canada). Platelets were then sedimented by centrifugation at 800g for 2 min and washed twice with the same buffer.

7. Protein Phosphorylation Studies.

Intact or digitonin permeabilized platelets previously labelled with [^{32}P]Pi were used in the experiments. When indicated and during the 5 min permeabilization period, aliquots of platelets were incubated alone or in the presence of 10 μM of either MPSD or Ala-MPSD. PMA or thrombin were present in the permeabilization media during the last 3 min. When total proteins (heat stable and heat sensitive) were studied, incubation was terminated by addition of an equal volume of twice

concentrated Laemmli's loading buffer (Tris-HCl, 125 mM; glycerol, 20%; SDS, 4%; 2β mercaptoethanol, 10%; bromophenol blue, 0.05%, pH 6.8) followed by incubation at 95°C for 7 min. When heat stable proteins were studied, incubation was stopped by addition of twice concentrated RIPA buffer (NaCl, 140 mM; KCl, 2.6 mM; K_2HPO_4, 10 mM; KH_2PO_4, 1.8; NP-40, 1%; sodium deoxycholate, 0.5%) containing 1 μg aprotinin/ml, 1 μg leupeptin/ml, 1 mM PMSF, 1mM $NaVO_4$, 1 mM NaF and 50 mM benzamidine followed by boiling for 10 min. Boiled platelet extracts were then centrifuged at 16,000 g for 2 min. Supernatants thus obtained were mixed with equal volumes of twice concentrated Laemmli's loading buffer. The preparations were then heated to 95°C for 7 min.

8. Electrophoresis and Immunoblotting.

(i) Sodium dodecyl sulphate-polyacrylamide gel electrophoresis (SDS-PAGE)

All protein samples were analyzed by monodimensional10% sodium SDS-PAGE according to the method of Doucet and Trifaró (1988). Gels were prepared from stock solutions of 25% acrylamide and 0.25% N,N'methylenebisacrylamide. The final composition of the separating gel was 10% acrylamide, 0.1% N,N'methylenebisacrylamide. 0.4% SDS, 5% glycerol, 100 mM Glycine and 200 mM Tris, pH 9.0. Polymerization was produced by addition of 0.1% ammonium persulphate and 0.05% TEMED prior to casting. Once the gel mixture was loaded, the surface was slowly covered with ethanol and the mixture was allowed to

polymerize for approximately 30 minutes. Once polymerized the stacking gel was layered on top of the separating gel. The stacking gel contained 4% acrylamide, 0.04% N,N'methylenebisacrylamide 0.4% SDS, 5% glycerol,4 mM EDTA and 70 mM tris-HCl and was degassed for 15 minuets prior to adding 0.1% ammonium persulphate and 0.005% TEMED. The stacking gel was allowed to polymerize for 60 minutes before samples were loaded.

Once the gel was loaded with samples, 600 ml of the running buffer (mM: Tris-HCl, 100; Glycine, 150; SDS, 0.1) was poured into the upper tank. The lower tank contained 1200 ml of the same buffer. Gels were then run under the constant current condition of 25 mA/gel using a GIBCO-BRL Electrophoresis power supply (Life technologies Inc., Gaithersburg, MD, USA).

(ii) Coomassie Blue Staining of SDS-PAGE gels.

Following electrophoresis some of the gels were stained for 30 min on a horizontal shaker with Coomassie Brilliant Blue (0.1% coomassie blue stain in 40% methanol and 7% acetic acid, R-250, BioRad, Mississauga, ON, Canada) to detect protein bands. The gels were then destained overnight in 25% methanol and 10% acetic acid and the placed on Model 583 gel drier filter paper (BioRad, Mississauga, ON, Canada) and covered with cellophane membrane (BioRad, Mississauga, ON, Canada). Gels were subsequently dried in a Gel Slab Dryer model 224 (BioRad, Mississauga, ON, Canada) for 1.5hr.

(iii) Immunoblotting of SDS-PAGE gels.

After SDS-PAGE, the gels were first soaked for 15 min in cold electrotransferring buffer (mM: Tris-HCl, 25; glycine, 150; and 20% methanol (v/v)). Proteins were electrotransferred from gels to nitrocellulose membranes (pore size: 0.45 μm, Bio-Rad, Mississauga, ON, Canada) for 1.5hr at 90V (setting 100% with water cooling; current increases from 0.8 to 1.4 A) in a LKB 2005 transfer electroblotting unit. The nitrocellulose membranes were first blocked with 5% Carnation non-fat dry milk in PBS for 1hr and then incubated overnight at 4°C with different primary antibodies (antibodies raised against different antigens, MARCKS, PKC, pleckstrin or tubulin), at the indicated dilutions in PBS containing 5% Carnation non-fat dry milk. The membranes were next washed three times, for 10 minuets each, with PBS containing 0.01% tween-20. Membranes were then incubated with the corresponding HRP-conjugated secondary antibodies (goat antimouse IgG, goat antirabbit IgG and rabbit antigoat IgG), at the indicated dilutions in PBS containing 5% Carnation non-fat dry milk, for 1.5hr at room temperature. The membranes were washed three more times with PBS containg 0.01% tween-20.

(iv) Detection of the immunoblotted proteins.

Protein bands were then visualized using Enhanced Chemo-Luminesence (ECL) reageant (Amersham, Oakville, ON, Canada) which is a light emitting non-radioactive method for detection of immobilized specific antigens conjugated directly or indirectly with horseradish peroxidase-labelled antibodies. Membranes were then

exposed to Hyperfilm™ ECL™ (Amersham, Oakville, ON, Canada), and the developed films were scanned for further analysis (see below).

9. Autoradiography and Densitometric Analysis.

Coomassie brilliant blue stained gels or nitrocellulose membranes obtained from the phosphorylation experiments were exposed to Hyperfilm™ ECL™ (Amersham, Oakville, ON, Canada). The intensity of the autoradiograph bands were analyzed using Scion Image Beta 2 software (Scion Corporation, Frederick, MD). The areas under the peaks were integrated using the same program and results were expressed in arbitrary units.

10. Fluorescence Microscopy.

Platelets were centrifuged at 1000 g for 10 min onto poly-L-lysine-coated glass slides using a bench top cytospin centrifuge (Cytofuge 2, Stat Spin Inc., Norwood, Mass., USA). Platelets were immediately fixed in 3.7% formaldehyde in PBS for 20 min. Preparations were washed several times with PBS, permeabilized with 1% Triton X-100 for 3 min, washed again with PBS and incubated with 1% BSA and 1% donkey pre-immune serum in PBS for 1h at room temperature to block non-specific binding sites. Platelets were then washed with PBS and incubated with either non-specific mouse IgG (control, 1:250 dilution), human MARCKS mouse

monoclonal antibody (1:250 dilution) or human CD41a mouse monoclonal antibody (1:200 dilution) for 1 h. at room temperature. All preparations were then washed three times with PBS and incubated for 1 h. with (secondary antibody) affinity purified CY^3-conjugated donkey Fab_2 fragment raised against mouse IgG (1:200 dilution). Preparations were then washed with PBS and mounted in Slowfade buffer containing 50% glycerol (Molecular Probes, Eugene, Oregon, USA). Preparations were examined using incident fluorescent light under a Zeiss Axoplan microscope equipped with an HBO 50 mercury lamp and an oil immersion objective (100x; 1.3 aperture). Pictures were taken with a Sony digital camera and the images were saved using a Northern Eclipse software (Empix, Mississauga, Ont., Canada). Images were then digitally imported into Adobe Photoshop software for further analysis. Images were printed on Epson quality paper using an Epson Stylus Photo EX colour printer (Epson America Inc., Torrance, CA, USA).

11. Protein assay.

The amount of protein present in cell lysates was measured using Bradford method (Bradford, 1976). Bradford method is a dye binding assay in which a differential color change of a dye occurs in response to various concentrations of protein. The absorbance maximum for an acidic solution of Coomassie™ Brilliant

Blue G-250 dye shifts from 465 nm to 595 nm when binding to protein occurs. The dye binds to primarily basic and aromatic amino acid residues, especially arginine. The extinction coefficient of a dye-albumin complex was found to be constant over a 10-fold concentration range. Different dilutions of cell lysates was prepared, mixed with Bio-Rad Protein Assay reagent and absorbance of samples was then measured at wave length of 595 nm in a spectrophotometer. Protein concentrations in the samples were calculated using a standard curve prepared with known amounts of purified bovine serum albumin.

12. Statistical analysis of the data.

All the data were analysed and values of mean and standard error were calculated using Corel Quattro Pro softwear (version 8). Statistical Graphs were prepared using Sigma Plot soft ware (version 5). In some cases, significance of differences among results were determined using unpaired Student t-test. The word "significant" refers only to a statistical difference with P value is equal to or less than 0.05 ($P \leq 0.05$).

Chapter (III)
Results

A. Characterization of the permeabilized platelets preparation.

1. Effects of permeabilization condition on PMA-induced platelet aggregation.

Intact platelets were resuspended in Locke's solution whereas digitonin permeabilized platelets were resuspended in K^+-glutamate buffer. Platelets were permeabilized by incubation for 5 min with 15 μM digitonin at room temperature in K^+-glutamate buffer as described in Materials and Methods. Aliquots (0.5 ml) of intact or permeabilized platelets suspensions were placed in siliconized glass cuvettes and let to equilibrate for 2 min at 37°C with stirring in a whole blood dual channel aggregometer. Ten microliters of either vehicle solution or a solution containing Phorbol 12-Myristate 13-Acetate (PMA, 100 nM final concentration) were then added to the corresponding preparation and platelet aggregation was monitored by measuring the change in light transmission as indicated in Materials and Methods. Under these conditions, PMA was found to induce aggregation only in intact platelets providing sufficient free Ca^{2+} (2.2 mM) was present in the medium. Neither intact platelets suspended in Ca^{2+}-free Locke's solution, nor permeabilized platelets (in presence and absence of Ca^{2+}) showed any aggregation in response to PMA (Fig 3.1).

2. Leakage of [^{3}H]5-HT from platelets during permeabilization.

In order to rule out the possibility that treatment with digitonin may cause leakage of [^{3}H]5-HT from platelet intracellular stores, the following experiment was

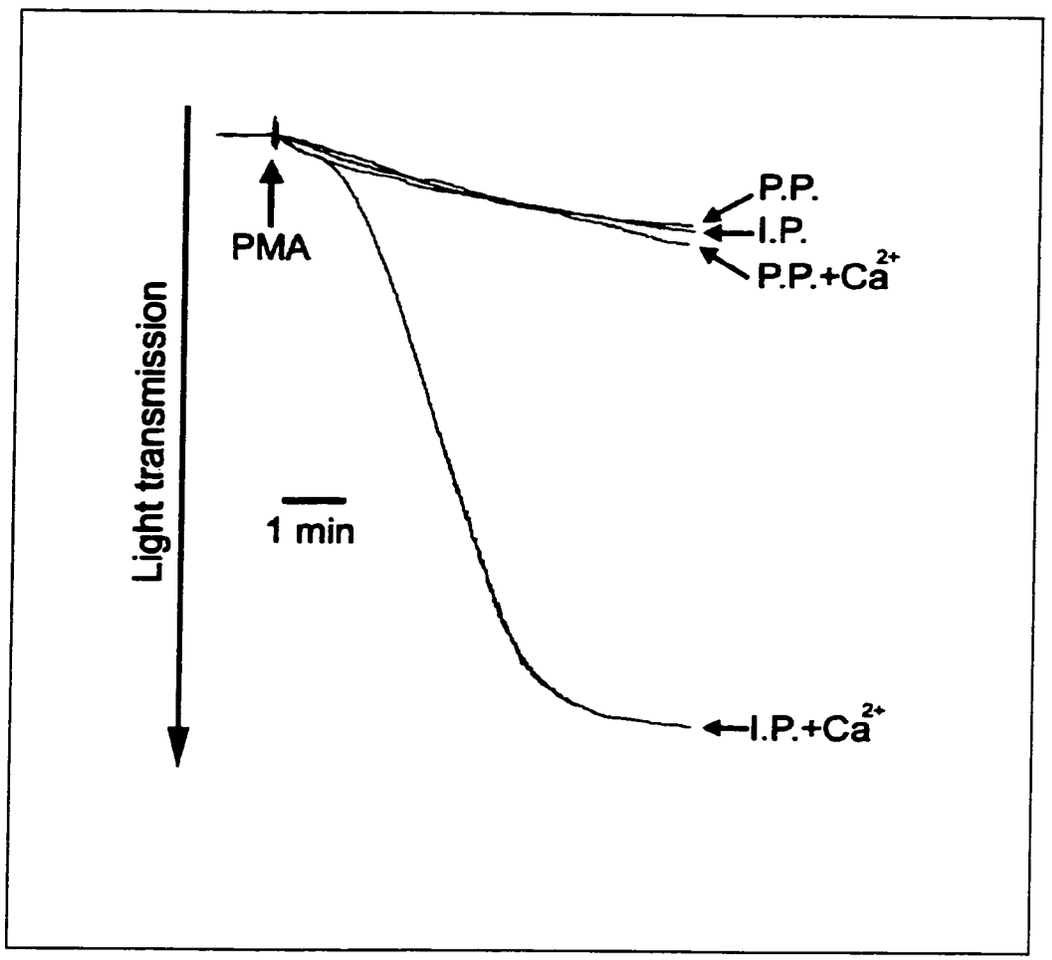

Fig 3.1. Effects of digitonin permeabilization on PMA-evoked platelet aggregation. Intact platelets (I.P.) were resuspended in regular Locke's or in Ca^{2+}-free Locke's solution whereas digitonin permeabilized platelets (P.P.) were resuspended in K^+-glutamate buffer either in the presence or absence of Ca^{2+}. Five hundred microliter platelet aliquots (7.5×10^8 platelets/ml) were placed in siliconized glass cuvettes under constant stirring. Platelet preparations were then challenged with 100 nM PMA and aggregation was monitored as described in Materials and Methods. Aggregation was observed only when I.P. were incubated in the presence of Ca^{2+}.

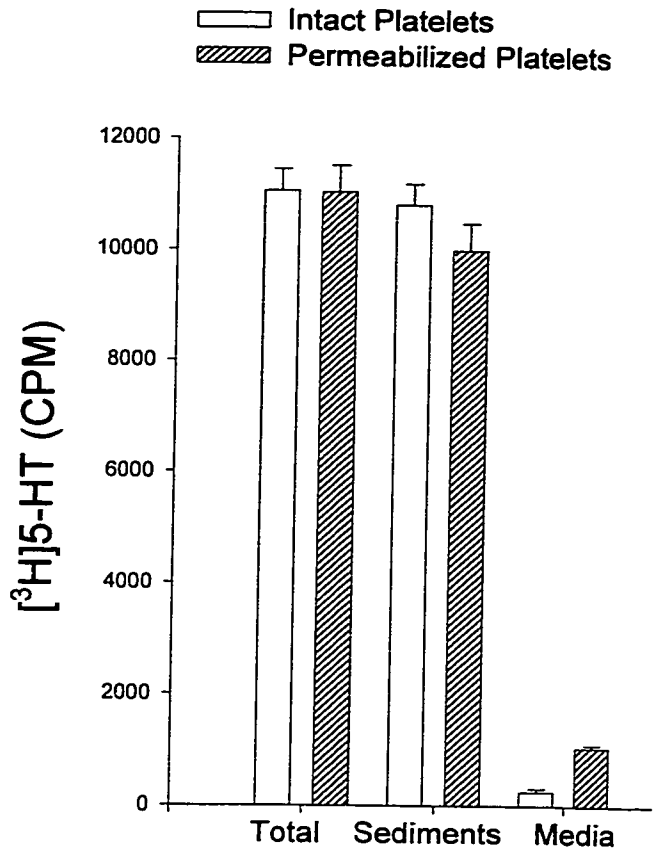

Fig 3.2. Effect of digitonin permeabilization on leakage of [^{3}H]5-HT from platelets. [^{3}H]5-HT-labelled platelets were incubated for 5 minutes either in Ca^{2+}-free Locke's solution or in K^+-glutamate buffer containing 15 μM digitonin. At the end of the incubation period platelets were recovered by centrifugation and radioactivity present in the media and in platelets (sediments) were measured as described in Materials and Methods. Total [^{3}H]5-HT content was calculated by adding the amount of radioactivity present in the media to that present in the platelet sediments. Bars represent the mean ± SEM of results obtained from 8 different preparations.

performed. Platelets previously labelled with [^{3}H]5-HT were incubated for 5 minutes either in Ca^{2+}-free Locke's solution or in K^+-glutamate buffer in the presence of 15 μM digitonin . At the end of the incubation period, preparations were centrifuged and sediments and supernatants were separated. Sediments were acid-digested and radioactivity present in both, sediments (platelets) and media, was measured as described under Materials and Methods. A very small amount of radioactivity was found in the medium after permeabilization (Fig 3.2) suggesting that serotonin stores remain intact after digitonin treatment.

3. Time course of the secretory response of permeabilized platelets.

Platelets were permeabilized with 15 μM digitonin as indicated in Materials and Methods and incubated for different periods of time in the presence or absence of 100 nM PMA. Intact platelets were also incubated under similar conditions. The time course of the spontaneous serotonin release was quite similar for intact and permeabilized preparations with the exception of the 15 min incubation period where the spontaneous release was higher for permeabilized platelets (Fig 3.3). Time courses of serotonin release for permeabilized and intact platelets stimulated by PMA for different periods of time were also quite similar. Here again, release from permeabilized platelets after 15 min incubation with PMA was higher than for intact platelets (Fig 3.3). Platelets were also permeabilized by different periods of time and they were stimulated for 45 sec with 100 nM PMA. Under these conditions all

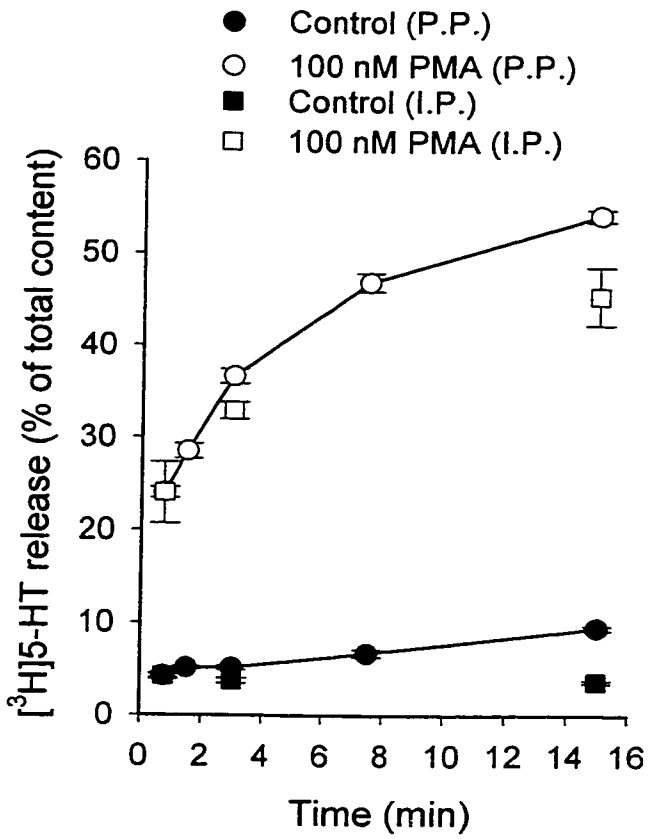

Fig 3.3. ***Time course of [^{3}H]5-HT output from intact (I.P.) and permeabilized platelets (P.P.) in the presence or absence of PMA.*** [^{3}H]5-HT labelled platelets were used and were permeabilized for different periods of time with 15 μM digitonin in K^+-glutamate buffer as indicated in Materials and Methods. When indicated, PMA (100 nM) was present during the entire incubation period. After different periods of incubation, platelets were recovered by centrifugation and the content of [^{3}H]5-HT was measured in the medium and in the platelets. [^{3}H]5-HT outputs were expressed as a percentage of total content. Values represent the mean ± SEM of at least 8 different preparations.

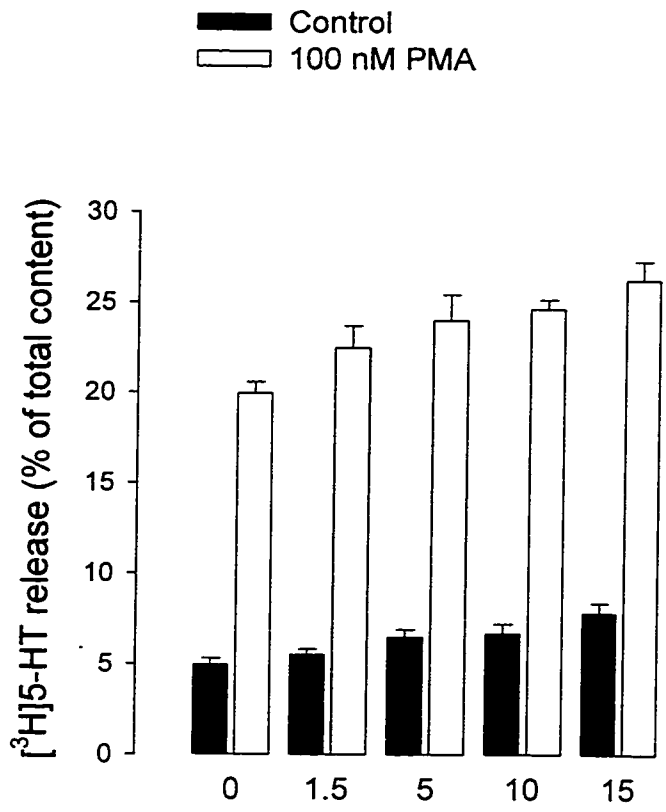

Fig 3.4. Effect of permeabilization for different periods of time on the responses to a short period of stimulation with PMA. [^{3}H]5-HT labeled platelets were permeabilized with 15 μM digitonin for different periods of time and they were stimulated for 45 s with 100 nM PMA. Platelets were recovered by centrifugation and [^{3}H]5-HT outputs were measured as indicated in Materials and Methods. Values were expressed as percentage of platelet total content. Bars represent mean±SEM of four different preparations.

secretory responses to PMA were similar (Fig 3.4).

4. Protein leakage from permeabilized platelets.

The effect of permeabilization on the leakage of proteins was also investigated. Permeabilized platelets were centrifuged after different periods of incubation with 15 μM digitonin in K^+-glutamate buffer. Platelets were then resuspended in volumes equal to that of the incubation media (supernatants). Equal aliquots of platelets suspensions and supernatants were taken and subjected to SDS-PAGE followed by immunoblotting using antibodies against PKC, pleckstrin and MARCKS (see next section for identification of MARCKS in platelets). PKC and pleckstrin were both detected in the medium under these conditions (Fig 3.5). However, MARCKS was not detected in the medium except when this was concentrated 30 times (Fig 3-5, lower panel). The content of PKC in the medium after 5 min treatment with digitonin was 24% of total content and 25% after 20 min. Similarly, the amount of pleckstrin in the medium was 19% and 28% after 5 and 20 min treatment with digitonin, respectively. Therefore, most of the protein leakage occurred during the first 5 min of permeabilization. In the case of MARCKS, the leakage into the medium corresponded to 2% and 4% of the total content after 5 and 20 min of permeabilization, respectively. Furthermore, the degree of digitonin permeabilization was monitored during the experiments using rhodamine-phalloidin (a probe for filamentous actin) as indicated in Materials and Methods. After 5 and 20 min of digitonin treatment, the

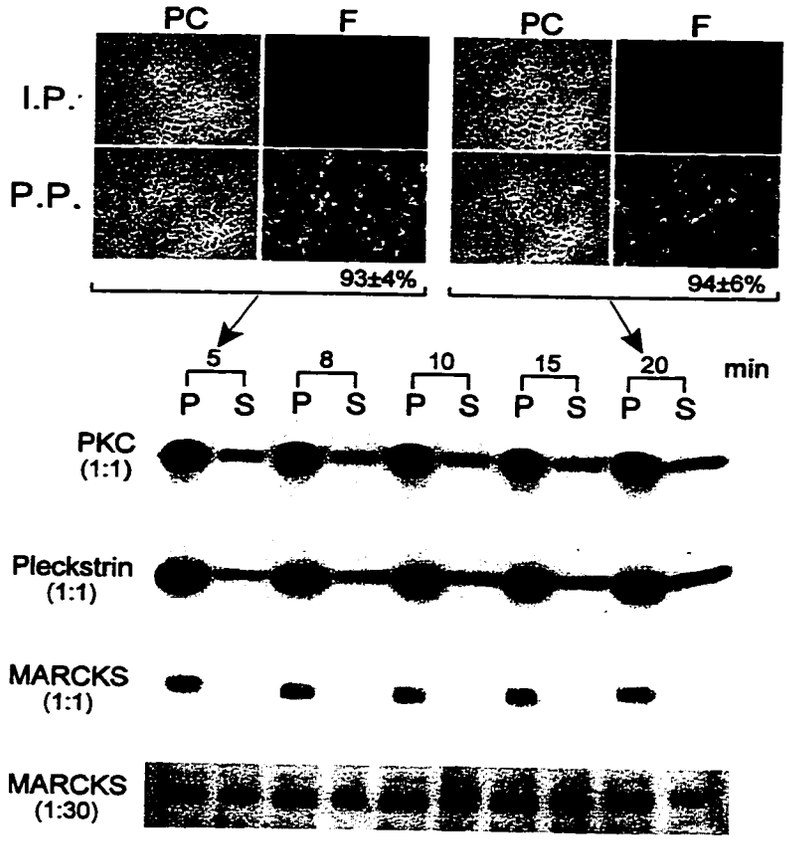

Fig 3.5. ***Leakage of proteins from digitonin permeabilized platelets.*** Platelets were incubated with 15 μM digitonin in K^+-glutamate buffer for different periods of time. Platelets were then recovered by centrifugation and pellets (P) were resuspended in K^+-glutamate buffer to the same volumes of the corresponding supernatants (S; incubation media). Equal aliquots (1:1) of P and S were run on SDS-PAGE followed by immunoblotting with antibodies against PKC, pleckstrin and MARCKS. Under these conditions, MARCKS was not detected in the medium (S). Therefore S was concentrated 30 times; aliquots were run again and tested for the presence of MARCKS (1:30). In this case a strong band was observed and this is shown at the bottom of the figure. The degree of platelet permeabilization in this experiment was determined by incubating fixed platelets for 15 min with rhodamine phalloidin (a probe for filamentous actin) as indicated in Materials and Methods. Results obtained for intact (I.P.) and permeabilized (P.P.) platelets after 5 and 20 min digitonin treatment are shown at the top of the figure. Phase contrast (PC) and fluorescence (F) fields of the preparations are shown at left and right, respectively.

percentages of permeabilized platelets were 93±4% (n=3) and 94±6% (n=3), respectively (Fig 3.5). This rules out the possibility that platelets were resealed during the 20 min incubation period.

B. Identification of MARCKS in platelets and its phosphorylation during PKC activation.

Platelets were sedimented on glass slides using a cytospin centrifuge as described in Materials and Methods and they were immunostained using a non-specific mouse IgG (as negative control) and mouse monoclonal antibodies against MARCKS and CD41a (fibrinogen receptor, the later as positive control). Platelets reacted with both antibodies and showed a strong fluorescence indicating the presence of both antigens (Fig 3.5 D and F). The fact that MARCKS is a major PKC substrate in other cell types suggests that PKC activation in platelets by PMA should lead to increase in MARCKS phosphorylation. Permeabilized platelets, previously labelled with [^{32}P]Pi, were also incubated for 3 min in the absence or presence of 100 nM PMA. Heat-stable proteins prepared as indicated in Materials and Methods were separated by 10% SDS-PAGE. Proteins were then electrotransferred to nitrocellulose membranes and this was followed by autoradiography and immunoblotting with a mouse monoclonal antibody raised against the C-terminal domain of human MARCKS. PMA treatment increased the phosphorylation of several heat stable proteins (Fig 3.7, lane 2). Two of these bands were identified as MARCKS by the

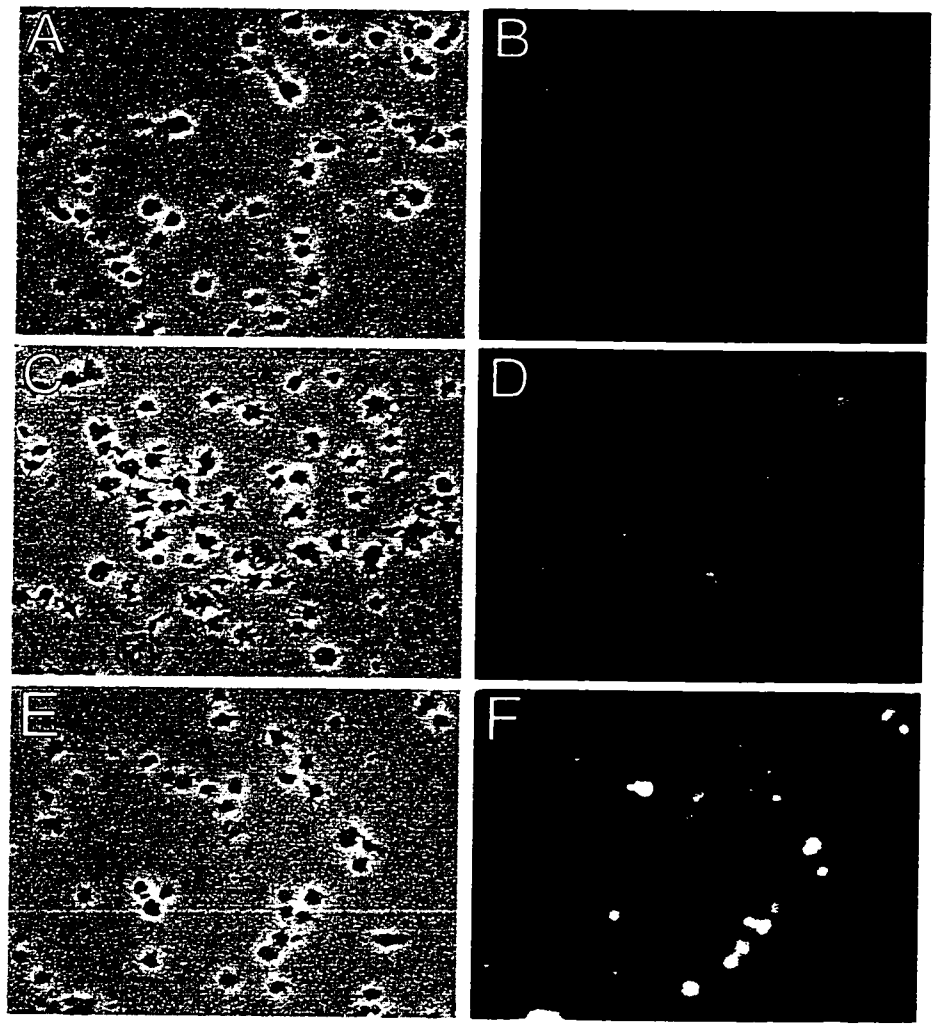

Fig 3.6. ***Immunostaining of platelets with MARCKS and CD41a antibodies.*** Intact platelets were fixed, permeabilized and immunostained with either non-specific mouse IgG (A and B), human MARCKS mouse monoclonal antibody (C and D) or human CD41a (fibrinogen receptor) mouse monoclonal antibody (E and F). The second antibody used was affinity purified Cy^3-conjugated donkey Fab_2 fragment raised against mouse IgG (dilution 1:200). Phase contrast is shown in A, C and E and fluorescence fields of the preparations are shown in B, D and F.

antibody (Fig3.7). MARCKS always appears on SDS-PAGE gels as a double band (Blackshear et al, 1986; Kligman and Patel, 1986; Patel at al, 1987). These bands had the same electrophoretic mobility of the MARCKS protein isolated from SH-SY5Y human neuroblastoma cells and the MEG-01 cell line (data not shown). There was an increase in the phosphorylation of MARCKS upon stimulation of platelets by PMA, indicated by the autoradiographs and the shift of MARCKS band on the immunoblot (Fig 3.7). In order to determine the relative abundance of MARCKS in platelets and because of the unavailability of purified MARCKS to develop a proper assay, we have used the MEG-01 cell line, a cell line derived from a megakaryocyte leaukemia (Ogura et al, 1985; Nagata et al, 1996), as positive control. Lysates of human platelets and MEG-01 cells were prepared by treatment with RIPA buffer, and the amount of protein present in the lysates was determined using the Bradford colorimetric method. Different amounts of protein were loaded onto each lane and they were subjected to 10% SDS-PAGE followed by a Western blot using a goat polyclonal antibody raised against a peptide mapping the C-terminus of human MARCKS. Densitometric scanning of the immuno-blots indicated that the concentration of MARCKS in platelets was 68±3% (n=4) of that in Meg-01 cells (Fig 3.8).

C. MARCKS Phosphorylation Site Domain (MPSD) Peptide blocks Serotonin Release induced by PKC Activation.

Because MARCKS is a major substrate of PKC, experiments were performed

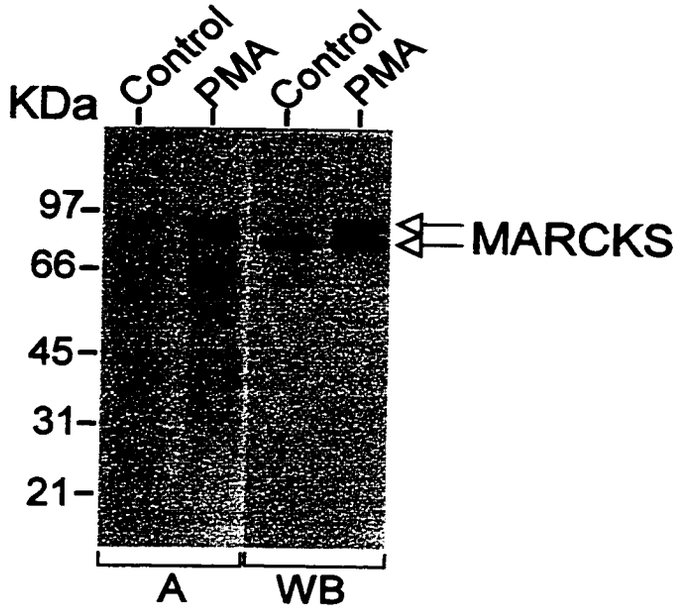

Fig 3.7. ***Detection of MARCKS in platelet extracts and its phosphorylation during protein kinase C (PKC) activation.*** Platelets labelled with [^{32}P]Pi as indicated in Materials and Methods were permeabilized with 15 μM digitonin in K^+-glutamate buffer for 5 min. During the last 3 min of permeabilization, 100 nM PMA in 0.05% DMSO (final concentration) or 0.05% DMSO (control) were present in the incubation medium. Reactions were stopped with RIPA buffer containing protease and phosphatase inhibitors (see Materials and Methods). SDS-PAGE was performed on boiled extracts of these preparations to separate heat-stable proteins. Proteins were electrotransferred to nitrocellulose membranes and autoradiography was first performed. This was followed by Western blotting with a mouse monoclonal antibody (4 μg/ml) raised against the C-terminal domain of human MARCKS. Two bands of 83 and 85 KDa were detected by the antibody; these corresponded to two phosphorylated protein bands found in the autoradiography. Although PMA increased the phosphorylation of both MARCKS bands, the upper band was heavily phosphorylated, increasing the amount of slow moving MARCKS (85 KDa) as indicated by a much heavier immunoreactivity of this band in the PMA-treated preparations.

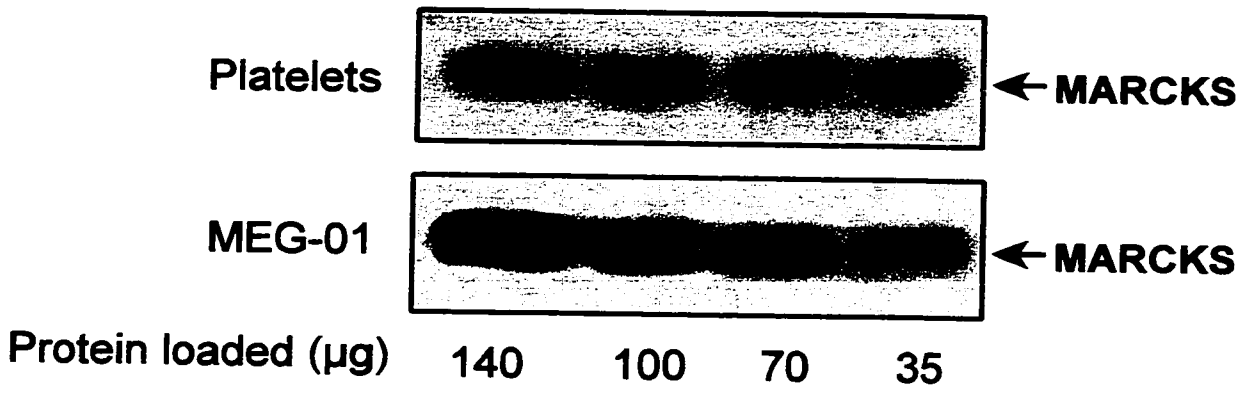

Fig 3.8. ***Comparison between the amount of MARCKS in platelets and MEG-01 cells.*** Equal protein aliquots of platelets and MEG-01 cells (a cell line derived from a megakaryocyte leukemia) were subjected to SDS-PAGE. Proteins were electrotransferred to nitrocellulose membrane and the immunoblot was performed with goat polyclonal antibody raised against a peptide mapping the C-terminus of human MARCKS. The numbers underneath the blot indicate the amount of protein loaded. Densitometric scanning of the immunoblot was performed as indicated in Materials and Methods.

to test the possibility of the involvement of MARCKS in the secretory response induced by PKC activation. The approach followed was the use of peptide MPSD of 25 amino acids with a sequence corresponding to the domain containing the phosphorylation site and also the calmodulin and actin binding sites (Fig 3.9) (Aderem, 1992). In addition, a similar 25 amino acid peptide, in which the serine residues were substituted by alanine (Ala-MPSD), was also tested (Fig 3.9). Permeabilized platelets were always stimulated for 45 sec. During this short period, not only a good secretory response to stimulation was obtained (Marcu, 1996) but this is also the time which, as shown in other well studied secretory systems (Vitale, 1995), is required for secretion from the release-ready vesicle pool (Vitale, 1995). PMA (100 nM), as expected, induced a significant ($P \leq 0.01$, $n = 24$) secretory response form permeabilized platelets (Fig 3.10). This increase in serotonin output was blocked in the presence of 10 μM MPSD ($P \leq 0.01$, $n = 24$). On the other hand, when Ala-MPSD was present at the same concentration, PMA-induced serotonin release was not affected (Fig 3.10). The inhibitory effect of MPSD on PMA-induced serotonin release was also concentration-dependent (Fig 3.11). Moreover, it is known that MPSD binds to calmodulin (CaM) and phosphatidylinositol 4,5-bisphosphate (PIP_2) and that its phosphorylation decreases the binding to these molecules. To rule out the possibility that the inhibitory effects of MPSD observed were due to either displacement or inhibition of CaM or PIP_2 effects, experiments were performed with these molecules. The presence in incubation medium of 10 μM of CaM or up to 100

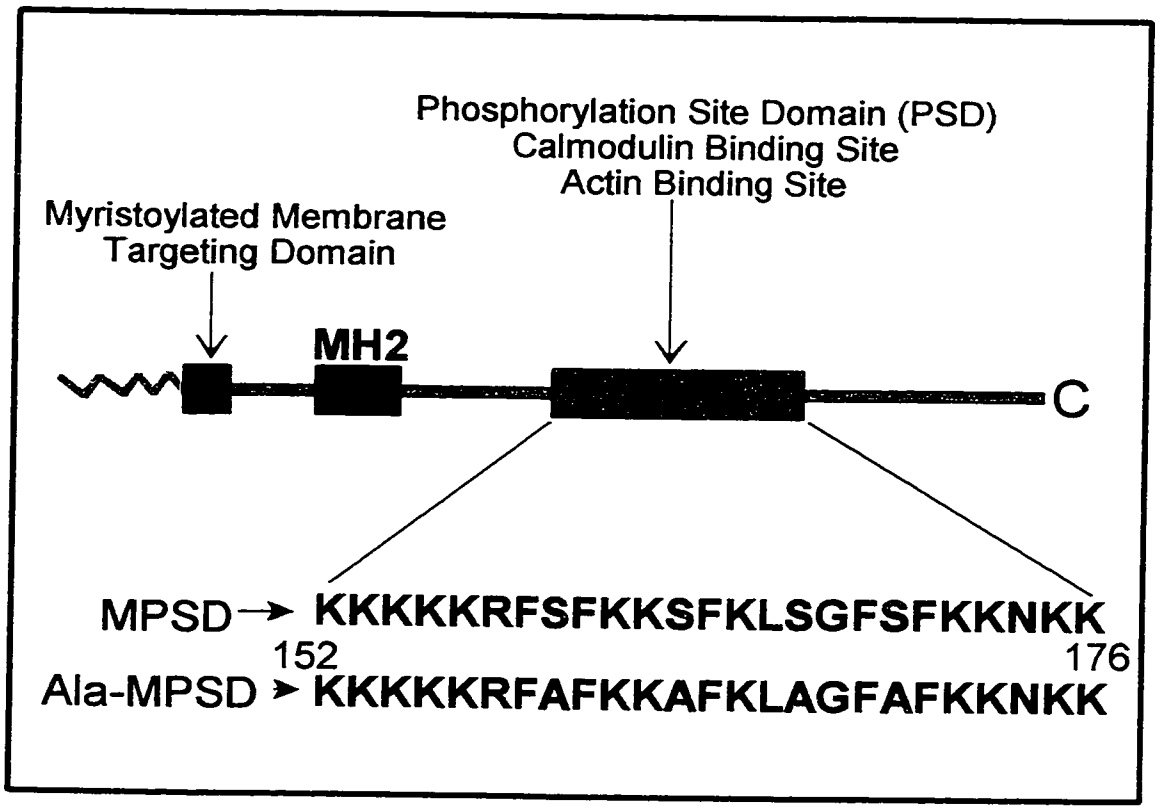

Fig 3.9. ***Structure of the MARCKS molecule and amino acid sequences of MPSD and Ala-MPSD peptides.*** MPSD has a 25 amino acid sequence corresponding to the phosphorylation site domain of MARCKS. In Ala-MPSD the four serine residues of MPSD have been substituted by alanines.

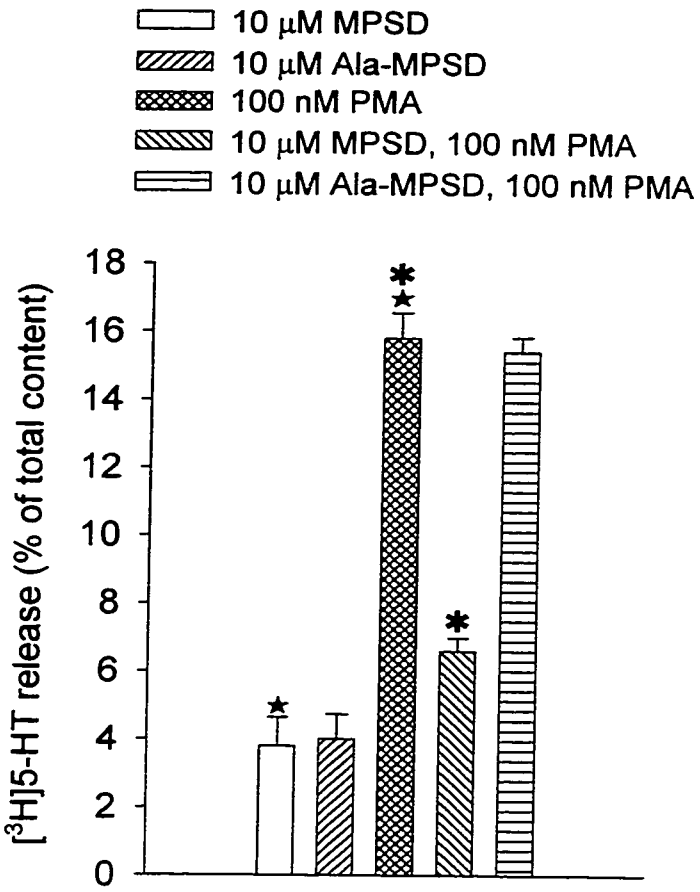

Fig 3.10. ***Effects of peptides MPSD and Ala-MPSD on PKC-induced [^{3}H]5-HT release from permeabilized platelets.*** [^{3}H]5-HT labelled platelets were permeabilized for 5 min with 15 μM digitonin in K^+-glutamate buffer as indicated in Materials and Methods. Permeabilization was done in the absence or presence of 10 μM of either peptide MPSD or peptide Ala-MPSD. Platelets were recovered by centrifugation and resuspended in the same medium for 45 s in absence or presence of 100 nM PMA. At the end of this stimulation period, [^{3}H]5-HT content was measured in the medium and in platelets. [^{3}H]5-HT outputs were expressed as percentage of total content after subtraction of spontaneous release. Each bar represents the mean ± SEM of results obtained from three different experiments. A minimum of 24 samples per condition were measured (★: $P \leq 0.01$, $n = 24$; ✱: $P \leq 0.01$, $n = 24$).

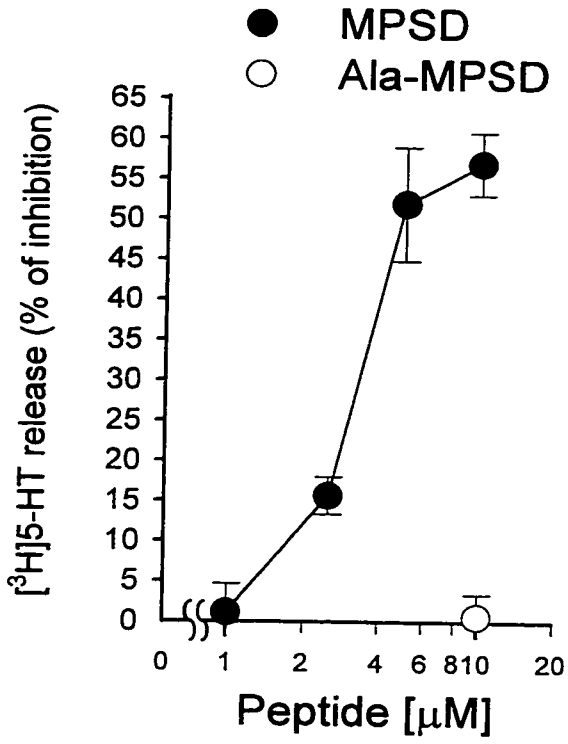

Fig 3.11. ***Concentration-dependent inhibition by MPSD of [^{3}H]5-HT output in response to PKC activation.*** Platelets were labelled with [^{3}H]5-HT, permeabilized with digitonin and incubated for 45 s with 100 nM PMA in the absence or presence of different concentrations (0, 1, 2.5, 5 and 10 μM)of either MPSD or Ala-MPSD as indicated in legend to Fig 3.10. Inhibition of PMA-induced [^{3}H]5-HT output was expressed as percentage of the [^{3}H]5-HT output in the presence of PMA alone. MPSD peptide shows a concentration-dependent inhibition of PMA-evoked [^{3}H]5-HT release and reached a plateau-like level at 10 μM (closed circles). Ala-MPSD at the higher concentration tested (10 μM) did not modify the secretory response induced by PMA (open circle). Values represent mean ± SEM of 8 different platelet preparations.

μM of PIP_2 neither modified serotonin spontaneous release nor affected or reversed the inhibition ($P \leq 0.01$, $n = 8$) by MPSD of PMA-induced serotonin release (Fig 3.12).

D. MARCKS Phosphorylation Site Domain (MPSD) Peptide partially inhibits Ca^{2+}-induced serotonin release and blocks PMA potentiation of Ca^{2+}-induced serotonin output.

Calcium and PMA were good platelet secretagogues in digitonin permeabilized platelets (Fig 3.13). When introduced into the incubation medium calcium and PMA had a very strong synergistic effects on serotonin release from permeabilized platelets ($P \leq 0.01$, $n = 8$) suggesting that these two stimuli might work through two distinct transduction pathways leading to platelet exocytosis. In addition to the observation that the MPSD peptide significantly blocked the PMA potentiation of Ca^{2+}-induced serotonin release ($P \leq 0.01$, $n = 8$) it also exerted some inhibitory effect on Ca^{2+}-evoked release of serotonin (Fig 3.13).

The fact that MPSD blocked PKC-induced release of serotonin suggests the involvement of MARCKS in the release reaction. This, together with the lack of effect of Ala-MPSD, also suggests that one or more of the serine residues present in MPSD are necessary for the inhibitory effect of the peptide. Because these serine residues are the phosphorylation sites of MARCKS as a result of PKC activation, the effects of MPSD and Ala-MPSD on platelet protein phosphorylation were investigated next.

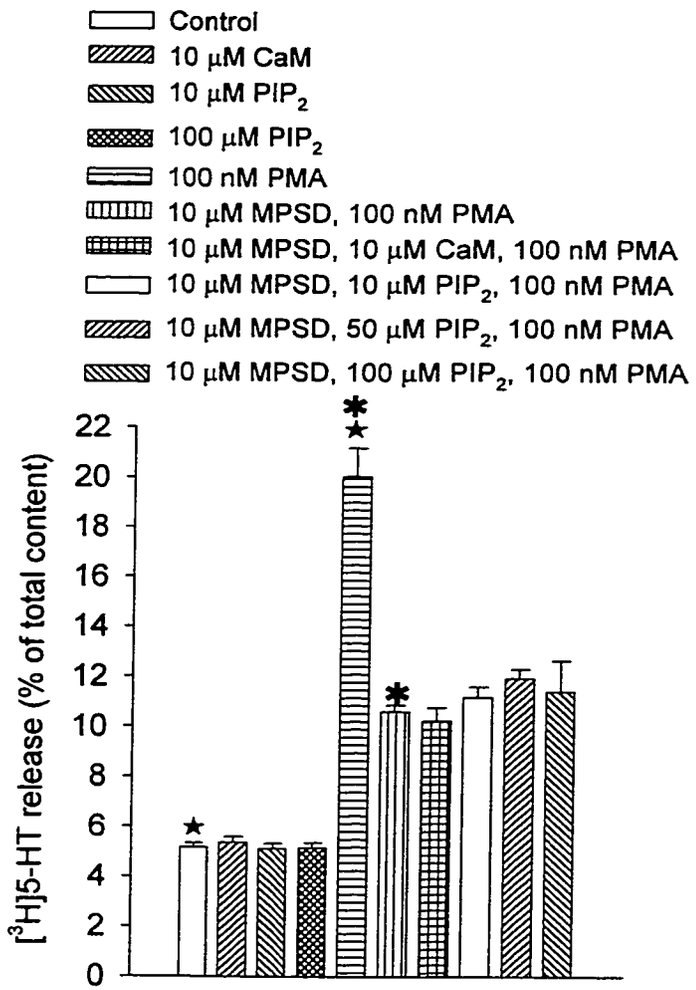

Fig 3.12. ***Effect of calmodulin (CaM) and phosphatydylinositol 4,5-bisphosphate (PIP_2) on the inhibition by MPSD of [^{3}H]5-HT output in response to PKC activation.*** Platelets were labelled with [^{3}H]5-HT, permeabilized with 15 μM digitonin and incubated with 100 nM PMA in the absence or presence of 10 μM MPSD. When indicated CaM or PIP_2 were present in the incubation medium. Values of [^{3}H]5-HT outputs were expressed as percentage of total content. Bars represent mean ± SEM of at least 8 different platelet preparations (★: $P \leq 0.01$, $n = 8$; ✱: $P \leq 0.01$, $n = 8$).

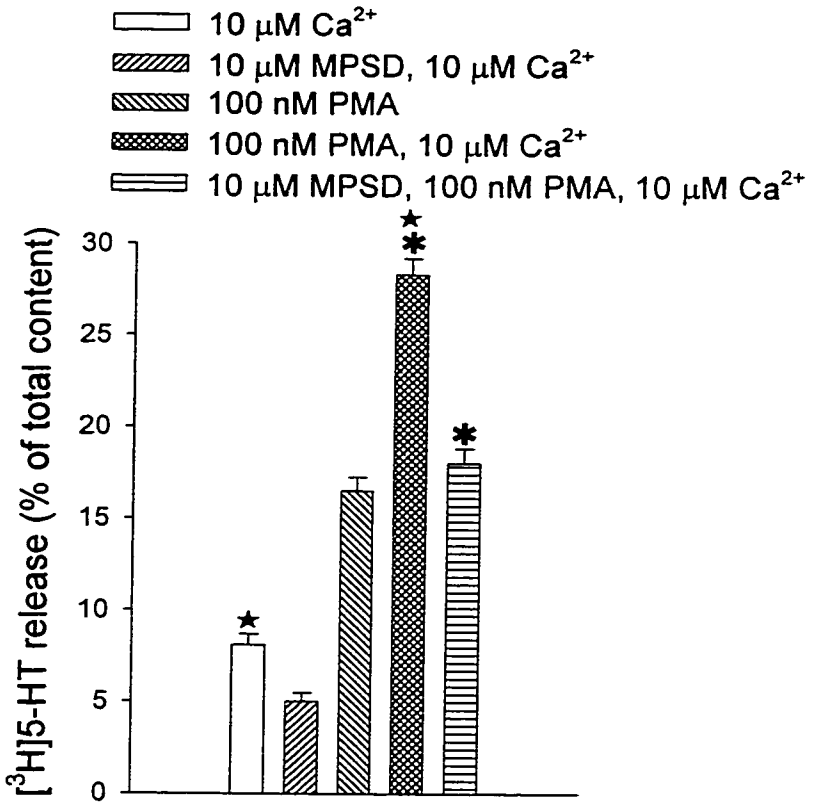

Fig 3.13. Effect of MPSD on the potentiation by PMA of Ca^{2+}-induced [^{3}H]5-HT release from permeabilized platelets. Platelets were labelled with [^{3}H]5-HT and permeabilized with 15 μM digitonin in presence or absence of 10 μM MPSD as indicated in Materials and Methods. When indicated, platelets were stimulated with either 10 μM Ca^{2+} or 10 μM Ca^{2+} plus 100 nM PMA. Bars represent mean ± SEM of 8 different preparations (★: P ≤0.01, n = 8; ✱: P ≤0.01, n = 8).

E. Effects of MPSD and Ala-MPSD on Protein Phosphorylation induced by PKC Activation.

Platelets previously labelled with [^{32}P]Pi were incubated with 100 nM PMA in the absence or presence of either MPSD or Ala-MPSD. This was followed by the separation of heat stable proteins by SDS-PAGE as described in Materials and Methods. A representative autoradiograph of four different experiments and its corresponding scannings are depicted in figure 3.14. Activation of PKC by PMA increased the phosphorylation of both MARCKS ($P \leq 0.05$, $n = 4$) and myosin light chain (MLC, $P \leq 0.05$, $n = 4$) as well as other unidentified heat-stable poly-peptides: p66, p50, p31 and p25 (Fig 3.14 and 3.15). Pleckstrin, a major PKC substrate is not heat-stable (see below). In the presence of 10 μM MPSD, there was a significant inhibition of PKC-induced phosphorylation of MARCKS ($P \leq 0.05$, $n = 4$, Fig 3.14, lanes 3 and 4). However, and as proof that PKC activity was intact under these conditions, there was no inhibition of PMA-induced phosphorylation of MLC (Fig 3.14, lane 4). Moreover, the phosphorylation of four other unidentified heat stable proteins increased upon PMA stimulation. Here again, there was no inhibition of this effect by MPSD (Fig 3.14, lane 4). Phosphorylations of p25, p31, p50 and p66 induced by PMA stimulation in the presence of MPSD were 102±7%, 104±4%, 99±8% and 95±7% respectively of phosphorylations induced by PMA in the absence of MARCKS inhibitor peptide. Further proof of PKC activation in the presence of MPSD was the fact that this peptide was phosphorylated (Fig 3.14, the asterisk). A

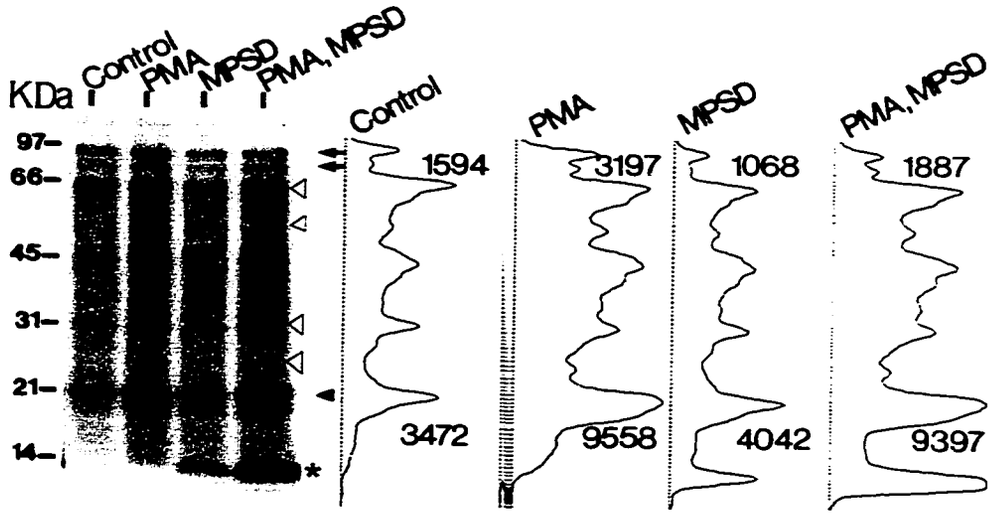

Fig 3.14. Effect of MPSD on the phosphorylation of MARCKS induced by PKC activation. Platelets were labelled with [^{32}P]Pi, permeabilized with digitonin in the absence or presence of 10 μM MPSD and subsequently stimulated as described in the legend to Fig 3.10. At the end of the stimulation periods, heat-stable platelet extracts were prepared and their proteins were separated by SDS-PAGE as described in Materials and Methods. Proteins were then electrotransferred to nitrocellulose membranes; these were exposed to Hyperfilm™ and the autoradiographies thus obtained were scanned as indicated in Materials and Methods. The figure shows the autoradiography of one such experiment. Double arrows show the position of MARCKS whereas single arrowheads indicate the position of myosin light chain (MLC). The open triangles indicate the position of proteins p25, p31, p50 and p66. The asterisk indicates the position of peptide MPSD which was less phosphorylated in the absence (basal PKC activity) than in the presence of PMA (basal + stimulated PKC activity). On the right-hand side is densitometric scannings of the autoradiograph. The numbers beside the MARCKS and MLC peaks are arbitrary units obtained from computer integration of peak areas. Similar results were obtained in 3 other experiments (see Fig 3.18).

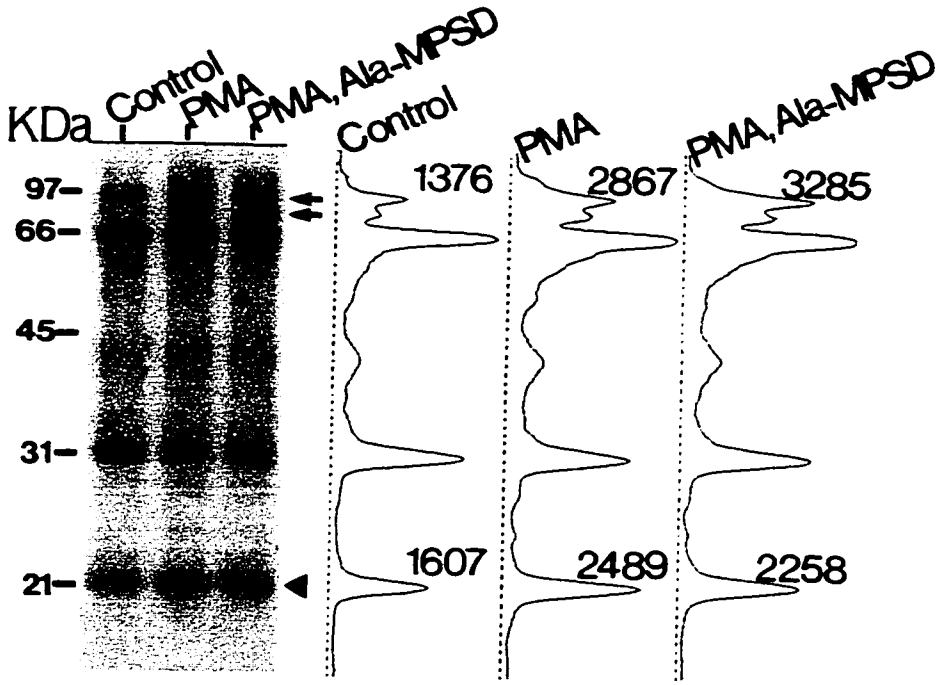

Fig 3.15. Effect of Ala-MPSD on the phosphorylation of MARCKS induced by PKC activation. Platelets were labelled with [^{32}P]Pi, permeabilized with digitonin in the absence or presence of 10 μM Ala-MPSD and subsequently stimulated as described in the legend to Fig 3.10. At the end of the stimulation periods, heat-stable platelet extracts were prepared and their proteins were separated by SDS-PAGE as described in Materials and Methods. Proteins were then electrotransferred to nitrocellulose membranes; these were exposed to Hyperfilm™ and the autoradiographies thus obtained were scanned as indicated in Materials and Methods. The figure shows the autoradiography of one such experiment. Double arrows show the position of MARCKS whereas single arrowhead indicates the position of myosin light chain (MLC). On the right-hand side is densitometric scannings of the autoradiograph. The numbers beside the MARCKS and MLC peaks are arbitrary units obtained from computer integration of peak areas. Similar results were obtained in 3 other experiments (see Fig 3.18).

much smaller level of phosphorylation of MPSD was observed in the absence of PMA (Fig 3.14, lane 4). Furthermore, when 10 μM Ala-MPSD was present in the medium, there was no inhibition of PKC-induced MARCKS phosphorylation (Fig 3.15). The increase in MLC phosphorylation was not affected and, as expected, Ala-MPSD was not phosphorylated (Fig 3.15). Because pleckstrin , a major PKC substrate, has been implicated in serotonin release (Yamada et al, 1987; Walker and Watson, 1993; Sloan and Haslam, 1997), it was important to determine the level of pleckstrin phosphorylation under conditions which PMA-induced serotonin release was blocked (i.e., presence of MPSD). Therefore, proteins present in unheated platelet extracts were separated by SDS-PAGE and the autoradiography and scanning of one such gel is shown in figure 3.16. The significant increase in the phosphorylation of pleckstrin ($P \leq 0.05$, $n = 4$) observed in the presence of 100 nM PMA was not modified with the presence of either MPSD or Ala-MPSD at a concentration at which MPSD inhibits MARCKS phosphorylation and serotonin release (Fig 3.16). Here again, the phosphorylation of MLC was not affected by the peptides and phosphorylation of MPSD was observed when PMA was present (Fig 3.16).

In order to test whether MPSD peptide enters the permeabilized platelets and that its PKC-induced phosphorylation takes place in the cytosol and not in the medium, as result of PKC leakage into the medium, a phosphorylation experiment was performed in which platelets were separated from the media by centrifugation at the end of the incubation time. Equal volumes of platelet sediments extracts and

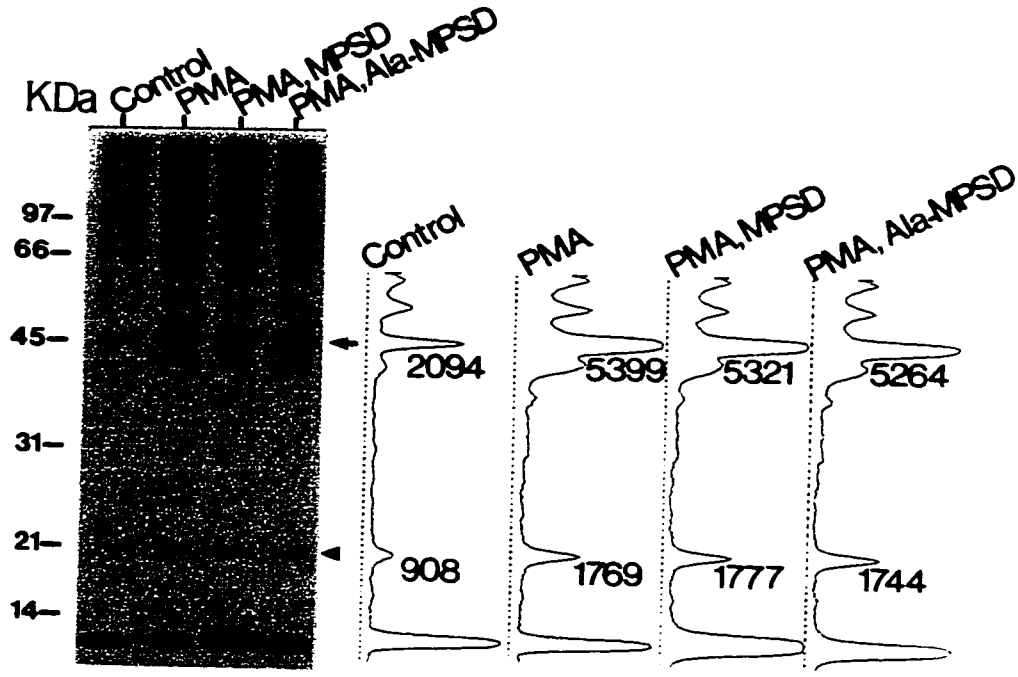

Fig 3.16. Effects of MPSD and Ala-MPSD on the phosphorylation of pleckstrin (p47) and myosin light chain (p20) induced by PKC activation. An experiment, similar to that described in the legend to Fig 3.14 was performed on [^{32}P]Pi labelled platelets, except that here, total platelet extracts (heat-stable plus heat-sensitive proteins) were prepared. SDS-PAGE, electrotransfers, autoradiography, scanning and integration of peak areas (arbitrary units) were performed as indicated in the legend to Fig 3.14 and in Materials and Methods. The arrow and the arrowhead indicate the position of pleckstrin and myosin light chain (MLC) respectively. The asterisk shows the position of phosphorylated MPSD which in this gel system migrated with another unknown phosphoprotein present in all lines. Similar results were obtained in other 4 experiments.

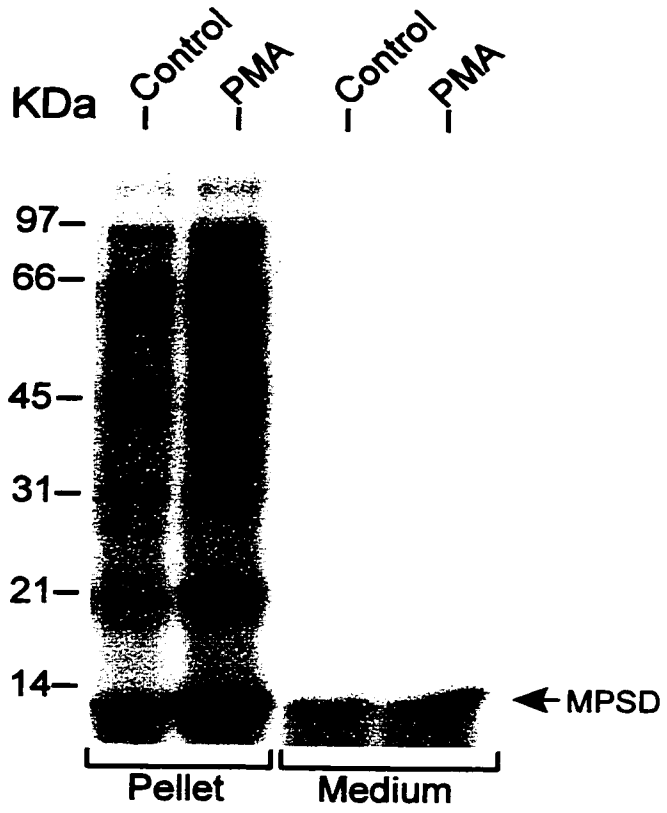

Fig 3.17. Leakage of phosphorylated MPSD from permeabilized platelets. Permeabilized platelets previously labelled with [^{32}P]Pi were incubated with 15 μM digitonin in the presence of 10 μM MPSD. When indicated platelets were stimulated with 100 nM PMA (lanes 2 and 4). Platelets were collected by centrifugation and sediments were resuspended in volumes equal to that of the incubation media. Equal aliquots of resuspended sediments and media were subjected to SDS-PAGE followed by autoradiography. It was necessary to expose the electrophoresis corresponding to the media twice as long as that of the sediments in order to detect some bands.

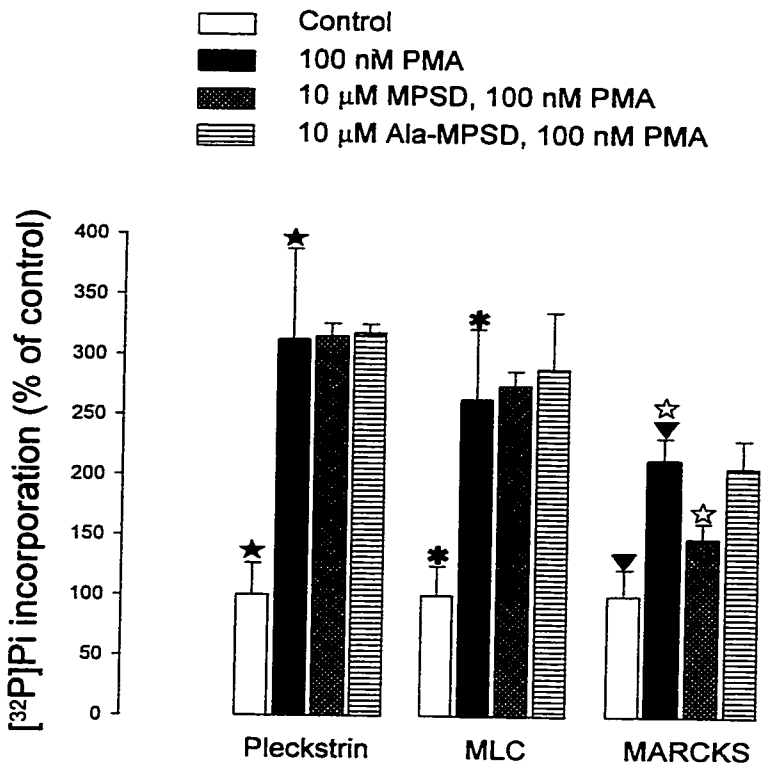

Fig 3.18. ***Cumulative data on protein phosphorylation obtained from experiments carried out on different permeabilized platelet preparations.*** Experiments were performed as described above in Fig 3.16 for pleckstrin and MLC and as indicated in legend to Fig 3.14 and 3.15 for MARCKS. Bars represent mean ± SEM of [^{32}P]Pi incorporation, expressed as percentage of control (absence of PMA), obtained from 4 different experiments for each condition tested (★: $P \leq 0.05$, $n = 4$; ✱: $P \leq 0.05$, $n = 4$; ▼: $P \leq 0.05$, $n = 4$; ☆: $P \leq 0.05$, $n = 4$).

media were subjected to SDS-PAGE followed by autoradiography. The autoradiographs (Fig 3.17) show that only very small amounts of phosphorylated platelet proteins leak out of the permeabilized platelets and that more than 90% of the MPSD peptide is phosphorylated in the cell interior. Moreover, it was necessary to expose to the film the membrane corresponding to the separation of proteins from the medium twice as long as that of the protein bands obtained from platelet sediments in order to detect some bands.

Figure 3-18 shows cumulative data from four different phosphorylation experiments. The levels of protein phosphorylations under each condition were determined from the autoradiographs by densitometric analysis as arbitrary units and expressed as percentage of control. Differences among groups were found to be significant ($P \leq 0.05$, $n = 4$) using unpaired Student t-test.

F. Similar PMA concentration-dependancy for MARCKS phosphorylation and serotonin release.

Serotonin output, pleckstrin and MARCKS phosphorylation were measured in platelets stimulated with increasing concentrations (1-500 nM) of PMA. Fig 3.19A shows one such phosphorylation experiment. Arrows indicate the first protein band for each type showing a significant increase in phosphorylation. Fig 3.19B shows cumulative data from 6 experiments. MARCKS phosphorylation and serotonin release curves were almost identical (EC_{50s} of 85 and 80 nM PMA respectively) whereas

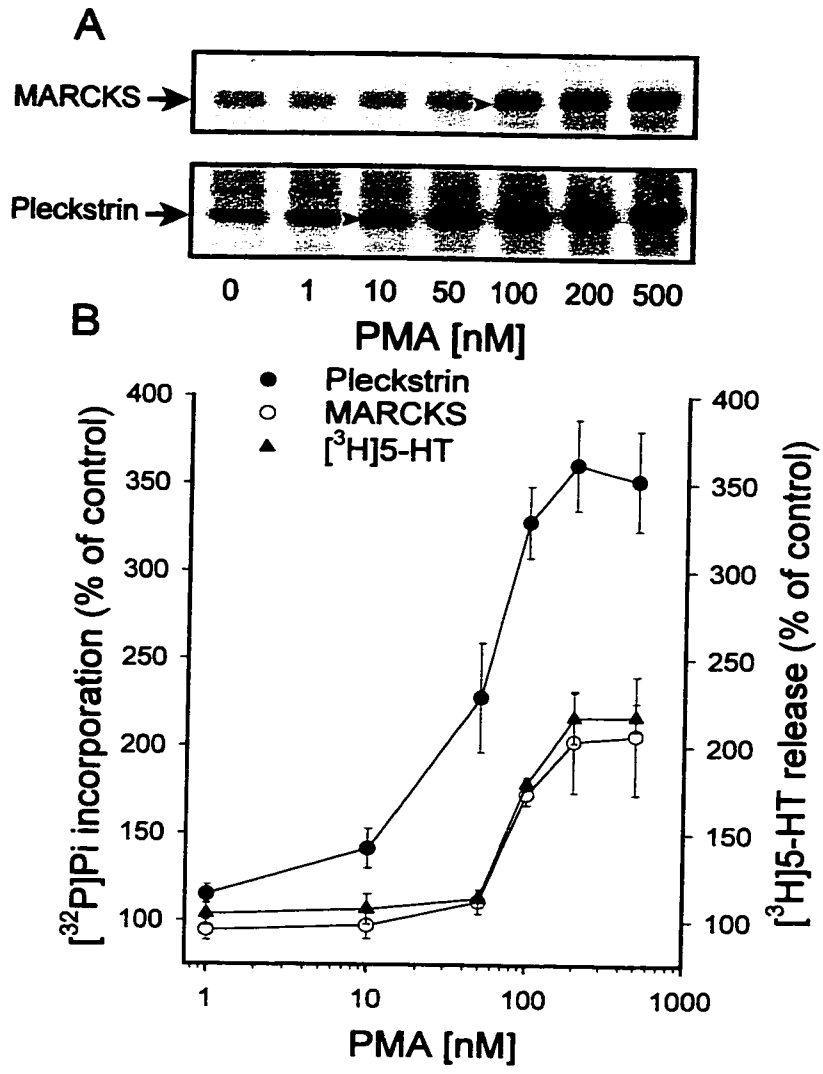

Fig 3.19. ***PMA concentration-dependent responses.*** Platelets were labelled with either [^{32}P]Pi or [^{3}H]5-HT, permeabilized with 15 μM digitonin and stimulated with increasing concentrations (1-500 nM) of PMA. [^{3}H]5-HT release and protein phosphorylation were measured as described in Materials and Methods. A) Autoradiography of SDS-PAGE gels of heat-stable platelet extracts (MARCKS) and whole platelet extracts (pleckstrin). The concentrations of PMA used are indicated at the bottom of the figure. Arrowheads indicate the smallest concentrations of PMA producing a significant increase in the phosphorylation of each protein. B) PMA concentration-dependent curves for [^{3}H]5-HT release and pleckstrin and MARCKS phosphorylation. Values represent the mean ± SEM of 6 different platelet preparations.

pleckstrin phosphorylation curve (EC_{50} of 45 nM PMA), although of similar shape to the other two, was shifted to the left. In other words, it was necessary to reach a concentration of PMA of 100 nM to observe significant ($P \leq 0.01$) and parallel increases in MARCKS phosphorylation ($n = 6$) and serotonin release ($n = 8$), whereas concentrations of PMA equal or lower than 50 nM significantly ($P \leq 0.01$) increased pleckstrin phosphorylation ($n = 6$, Fig 3.19B).

G. Effect of thrombin stimulation on MARCKS phosphorylation in intact platelets.

It is known that thrombin stimulation of intact platelets increases the phosphorylation of pleckstrin, an effect mediated through PKC activation (Haslam and Davidson, 1984; Hashimoto, 1987; Yamada et al, 1987; Kroll et al, 1993; Walker and Watson, 1993; Freedman et al, 1996; Hashimoto et al, 1997; Sloan and Haslam, 1997). Therefore, it is quite possible that thrombin stimulation might lead to an increase in the phosphorylation of MARCKS. Platelets previously labeled with [^{32}P]Pi were incubated with thrombin (1U/ml) and aggregation was monitored as indicated in Materials and Methods. At the point of maximal aggregation (Fig 3.20), heated and unheated protein extracts were prepared from platelets and proteins were separated by SDS-PAGE. Autoradiographs and scannings of one such experiment are shown in Fig 3.21 and Fig 3.22. As expected, thrombin stimulation significantly ($P \leq 0.01$, $n = 5$) increased the level of phosphorylation of pleckstrin and MLC (Fig 3.22 and Fig 3.23). Furthermore, the phosphorylation of MARCKS was increased ($P \leq 0.01$, $n =$

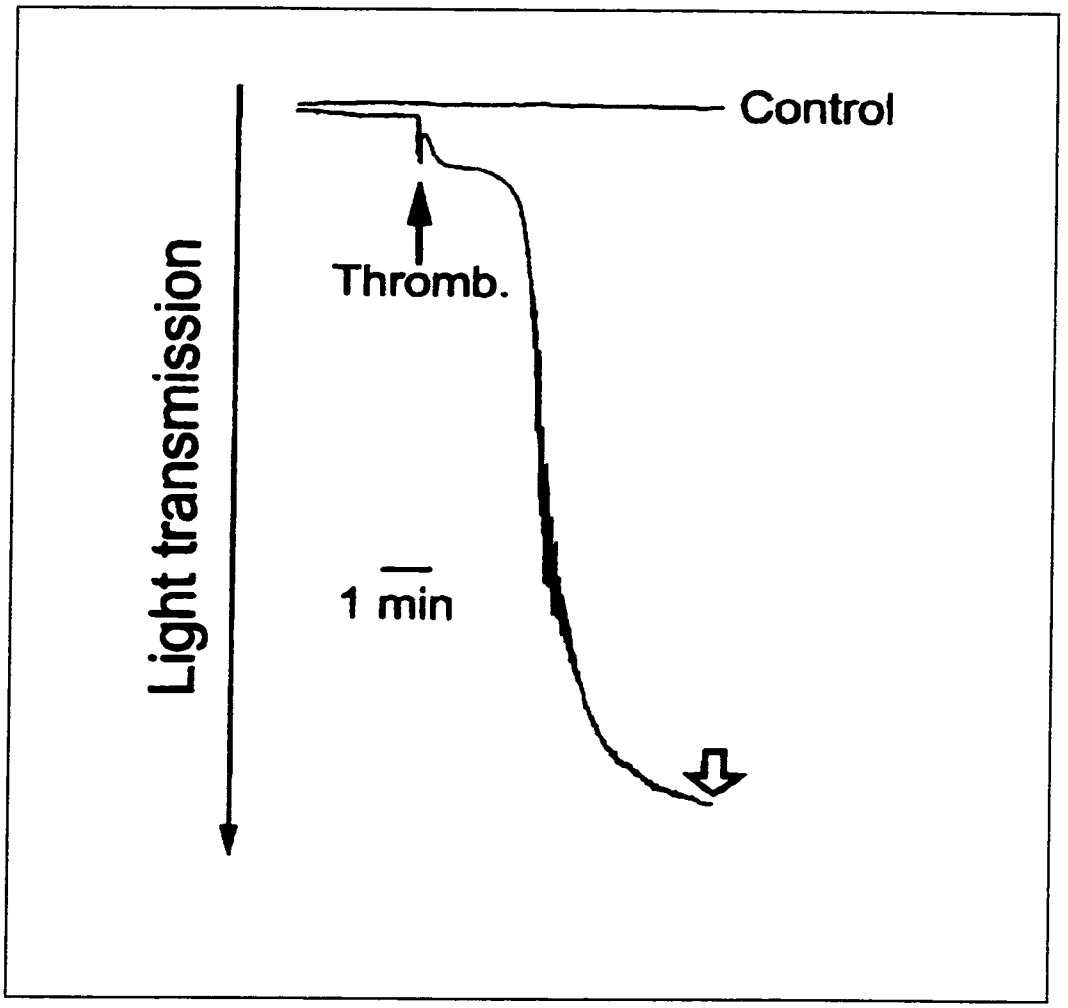

Fig 3.20. Platelet aggregation induced by thrombin stimulation. Aliquots (0.5 ml) of [^{32}P]Pi-labeled platelets suspended in Buffer P at the concentration of 7.5 × 10^8 platelets/ml were incubated in siliconized glass cuevettes placed in a whole blood aggregometer. Fifty microliters of either vehicle (control) or thrombin, to give final concentration of 1U thrombin/ml, were added and platelet aggregation was monitored following the increase in light transmission as described in Materials and Methods. At the point of maximum aggregation (open arrow), reactions were stopped and platelets extracts were prepared for further measurements (see legends to figures 3.21, 3.22 and 3.23).

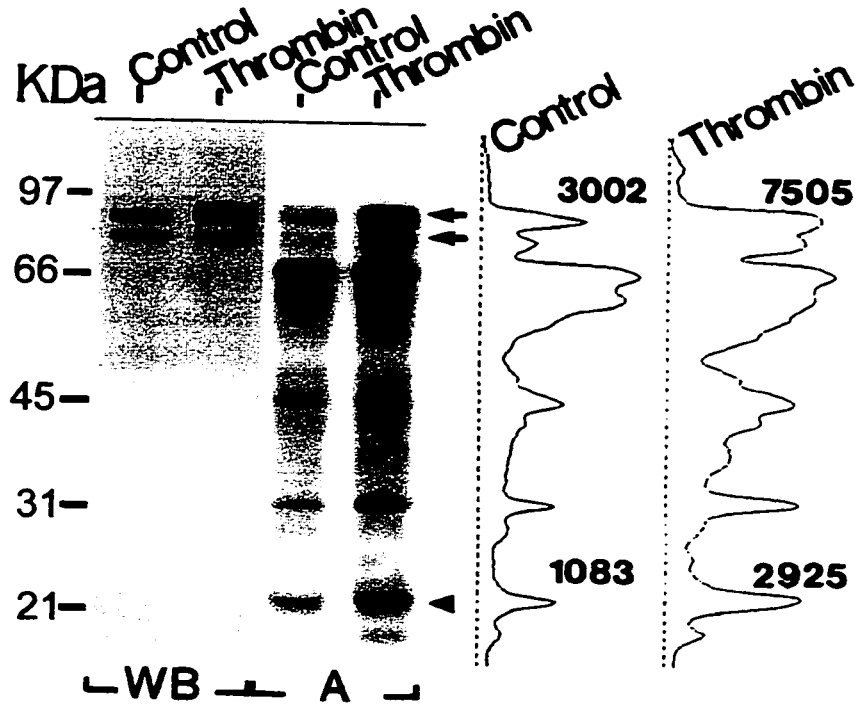

Fig 3.21. Thrombin-induced phosphorylation of MARCKS and other heat-stable proteins. Extracts of [^{32}P]-labeled platelets obtained at the point of maximum aggregation in response to thrombin (1U/ml, open arrow in Fig 3.20), were heated at 95 °C for 10 min and centrifuged for 2 min at 1600g. Supernatants containing heat-stable proteins were separated by SDS-PAGE and electrotransferred to nitrocelluse membranes. The membranes were first autoradiographed and then immunoblotted with a mouse monoclonal antibody raised against the C-terminal domain of human MARCKS. One such immunoblot and its corresponding autoradiograph as well as the densitometric scanning are shown here. The arrows indicated the position of MARCKS bands and the arrowhead shows the MLC band. Arbitrary units which represent values of peak areas are shown next to the peaks.

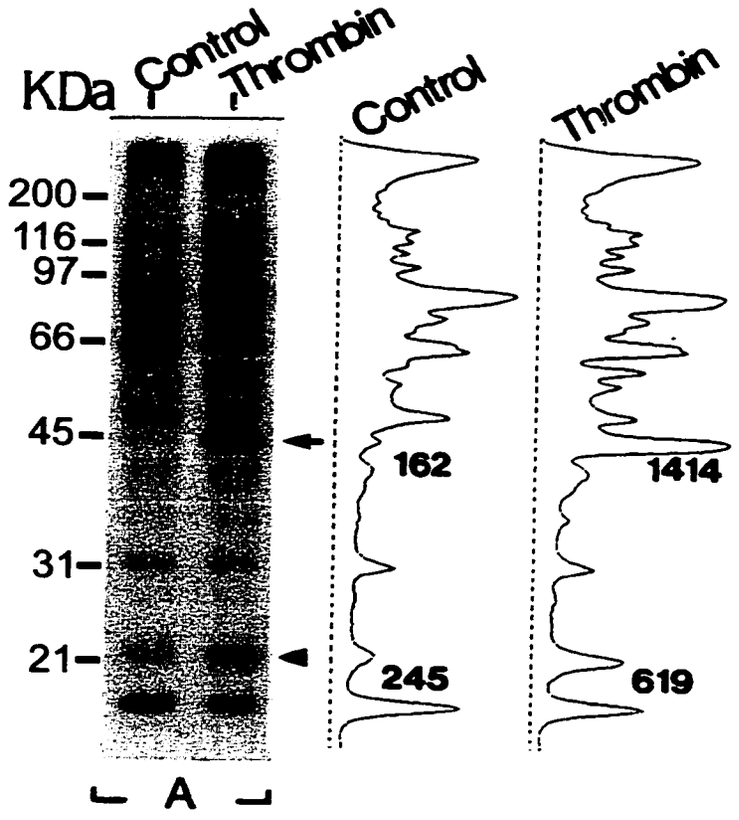

Fig 3.22. Thrombin-induced phosphorylation of platelet proteins. Extracts of [^{32}P]-labeled platelets obtained at the point of maximum aggregation induced by 1U thrombin/ml (Fig 3.20) were separated by SDS-PAGE. Gels thus obtained were stained with Coomassie Brilliant Blue, dried, autoradiographed, and scanned as described in Materials and Methods. One such autoradiograph and its corresponding densitometric scanning are depicted here. The arrow and arrowhead indicate the position of pleckstrin and MLC, respectively. Arbitrary units which represent values of peak areas are shown next to the peaks.

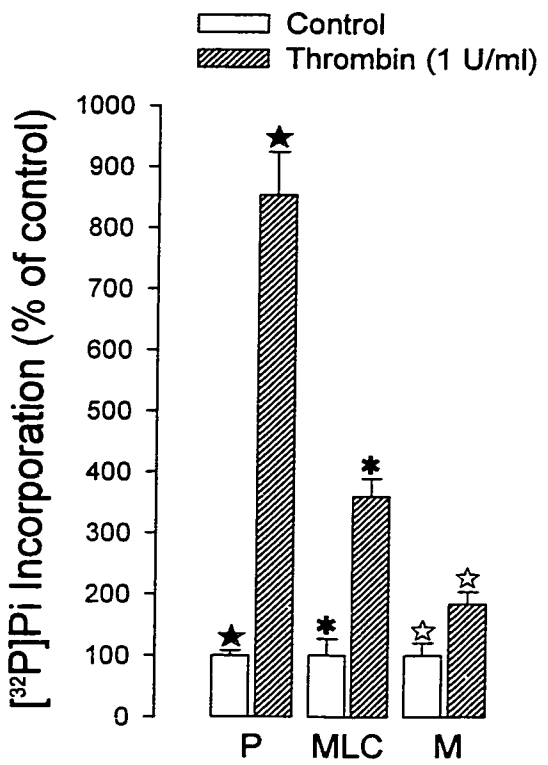

Fig 3.23. Cumulative data on thrombin-induced phosphorylation of pleckstrin, MLC and MARCKS. Experiments were performed as described above in Fig 3.22 for pleckstrin and MLC and as indicated in legend to Fig 3.21 for MARCKS. Bars represent mean ± SEM of [^{32}P]Pi incorporation, expressed as percentage of control (absence of thrombin), obtained from 5 different experiments for each condition tested (★: P≤0.01, n = 5; ✱: P≤0.01, n = 5; ☆: P≤.0.01, n = 5).

5) under these conditions (Fig 3.21 and Fig 3.23) suggesting that, as during PMA stimulation, MARCKS phosphorylation might mediate some of the thrombin-induced effects in platelets.

H. Similar thrombin concentration-dependency for MARCKS phosphorylation and serotonin release from intact platelets.

Experiments were performed to measure serotonin release, pleckstrin and MARCKS phosphorylation in platelets stimulated with increasing concentrations (0.001-1.0 U/ml) of thrombin. The experiments were performed on platelets suspended in Ca^{2+}-free Locke's solution. One such phosphorylation experiment is shown in Fig 3.24A. Arrows indicate the first protein band for each protein (MARCKS and pleckstrin) showing a significant ($P \leq 0.01$, $n = 5$) increase in phosphorylation. The degree of protein phosphorylation was quantified as described in Materials and Methods, and Fig 3.24B shows cumulative data from 5 experiments. MARCKS phosphorylation and serotonin release curves were almost identical whereas pleckstrin phosphorylation was shifted to the left. In other words, it was necessary to reach a concentration of thrombin of 0.05 U/ml to observe significant ($P \leq 0.01$) and parallel increases in MARCKS phosphorylation ($n = 5$) and serotonin release ($n = 8$), whereas concentrations of thrombin equal or lower than 0.01 U/ml significantly increased pleckstrin phosphorylation ($P \leq 0.01$, $n = 5$, Fig 3.24B).

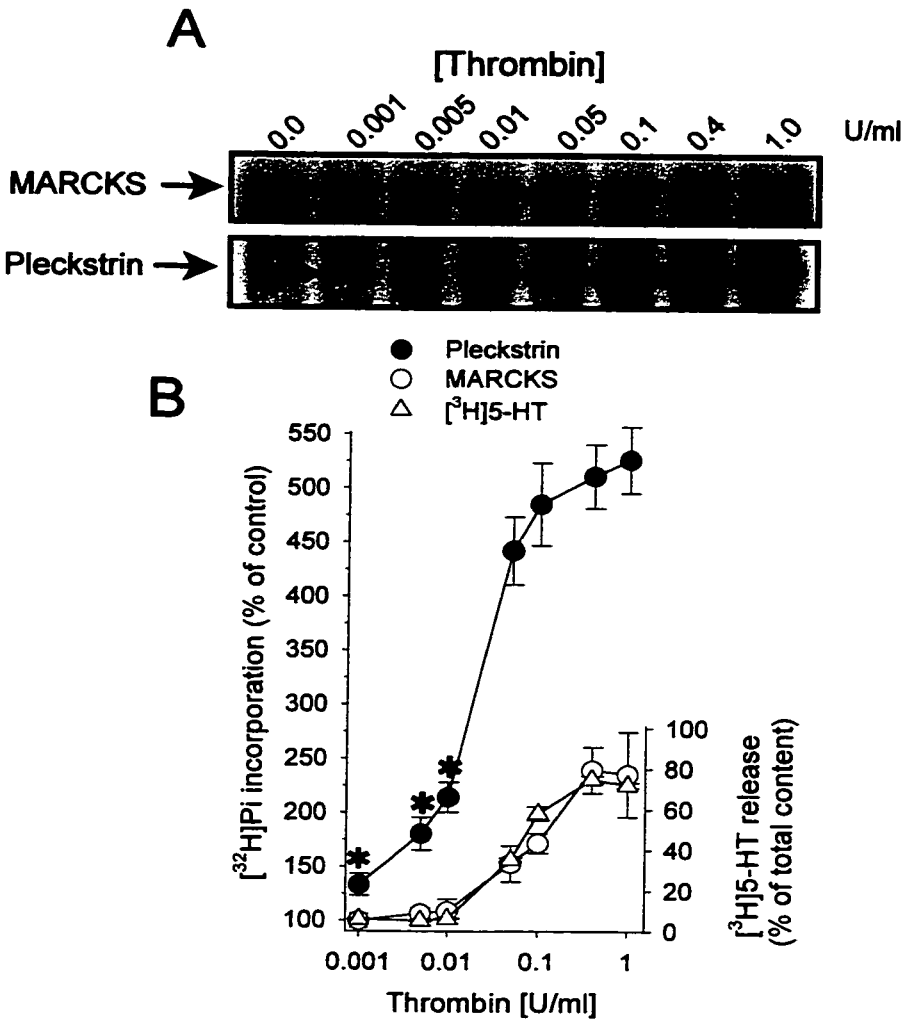

Fig 3.24. Thrombin concentration-dependent responses. Platelets were labeled with either [^{32}P]Pi or [^{3}H]5-HT as indicated in Materials and Methods and stimulated for three min with increasing concentrations (0.001-1 U/ml) of thrombin in Ca^{2+}-free Locke's solution. [^{3}H]5-HT release and protein phosphorylation were measured as described in Materials and Methods. A) Autoradiography of SDS-PAGE gels of heat-stable platelet extracts (MARCKS) and whole platelet extracts (pleckstrin). The concentrations of thrombin used are indicated at the top of the figure. Arrowheads indicate the smallest concentration of thrombin producing a significant increase in the phosphorylation of each protein. B) Thrombin concentration-dependent curves for [^{3}H]5-HT relase, and pleckstrin and MARCKS phosphorylation. Values represent the mean ± SEM of 5 different platelet preparations ($P \leq 0.01$, $n = 5$).

I. Effects of extracellular Ca^{2+} on thrombin-induced [^{3}H]5-HT release and protein phosphorylation in intact platelets.

The experiments with thrombin described in the previous sections were performed in Ca^{2+}-free solutions. Therefore, in order to assess the effect of extracellular Ca^{2+} in thrombin-induced platelet phosphorylation and serotonin release, additional experiments were carried out. Platelets were labelled with [^{3}H]5-HT and suspended either in Ca^{2+}-free Locke's solution or regular Locke's solution (2.2 mM Ca^{2+}). Aliquots were taken from each preparation and were incubated with increasing concentration of thrombin (0.01-1.0 U/ml). [^{3}H]5-HT outputs were then measured as described under Materials and Methods. Concentration-dependent curves of thrombin-induced [^{3}H]5-HT release from intact platelets both in presence and absence of extracellular Ca^{2+} were almost identical (Fig 3.25). As shown before (Haslam and Davidson 1984), the results suggest that thrombin-induced release of serotonin from intact platelets is independent of the availability of extracellular Ca^{2+}.

In other set of experiments, thrombin-induced phosphorylation of MARCKS, pleckstrin and MLC in presence or absence of extracellular Ca^{2+} were measured. Platelets were labelled with [^{32}P]Pi, as described under Materials and Methods, and suspended either in Ca^{2+}-free buffer P or in the same buffer containing 2.2 mM free Ca^{2+}. Aliquots were then taken and incubated with thrombin (1 U/ml) for 3 min. At the end of the incubation period, heat-stable platelet extracts and total platelet extracts

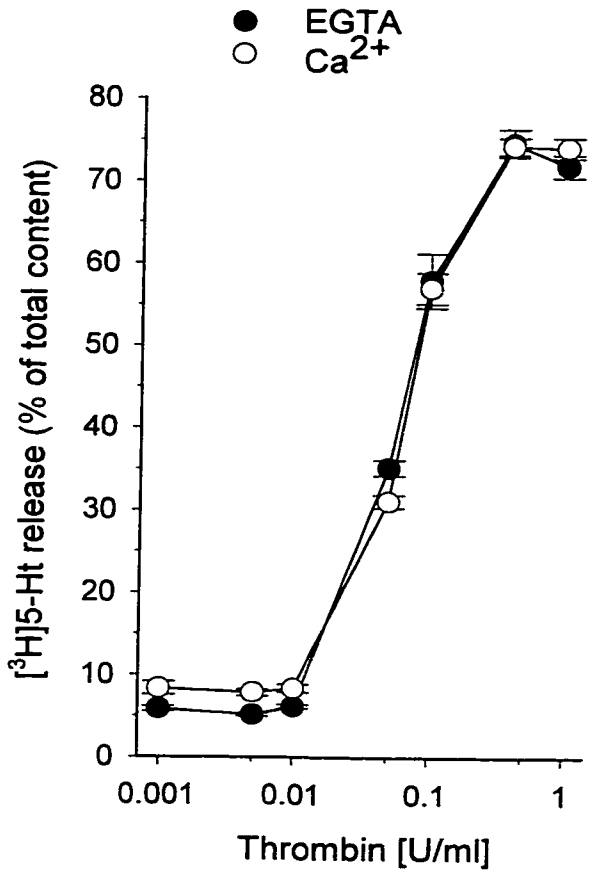

Fig 3.25. Effect of extracelluiar calcium on thrombin-induced [^{3}H]5-HT release from intact plateletrs. [^{3}H]5-HT-labelled platelets were suspended either in Ca^{2+}-free Locke's solution (close circles) or regular Locke's ssolution containing 2.2 mM free Ca^{2+} (open circles) and incubated for 3 minutes with different concentration of thrombin. At the end of this stimulation period, [^{3}H]5-HT content was measured in platelets as described in Materials and Methods. [^{3}H]5-HT outputs were expressed as percentage of total content. Values represent mean ± SEM of results obtained from eight different perparations.

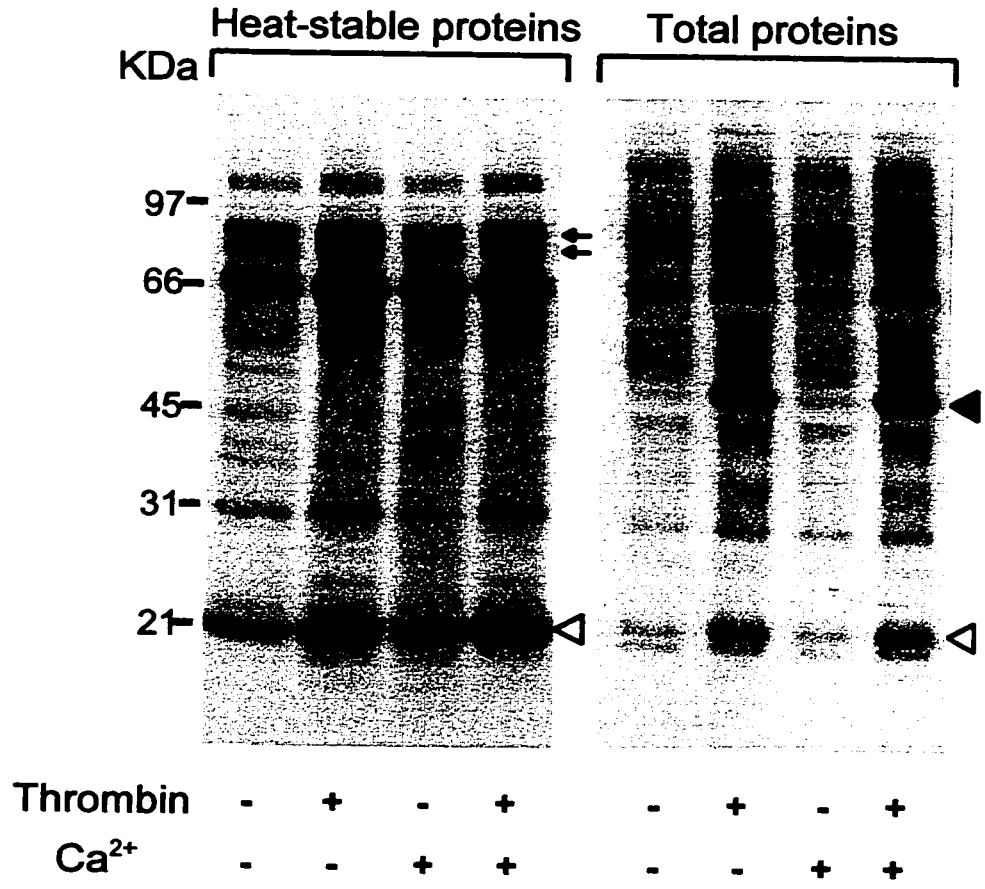

Fig 3.26. ***Effects of extracellular Ca^{2+} on thrombin-induced phosphorylation of MARCKS, pleckstrin and MLC.*** Intact platelets labelled with [^{32}P]Pi were suspended either in Ca^{2+}-free buffer P (0.5 mM EGTA) or in the same buffer containing 2.2 mM free Ca^{2+} and incubated for 3 min with either vehicle or 1 U thrombin/ml. At the end of the incubation period, heat-stable platelet extracts (left panel) and total platelet extracts (right panel) were prepared and subjected to SDS-PAGE followed by autoradiography. Arrows, closed arrowhead and open arrowheads indicate the position of MARCKS, pleckstrin and MLC, respectively.

were prepared and subjected to SDS-PAGE folowed by autoradiography as described in Materials and Methods. Thrombin-induced phosphorylation of MARCKS, pleckstrin and MLC, in intact platelets, were the same in presence or absence of extracellular Ca^{2+} (Fig 3.26).

J. Effect of digitonin permeabilization on thrombin-induced platelet aggregation and [^{3}H]5-HT release.

In order to use the peptides MPSD and Ala-MPSD to further study the role of MARCKS and its phosphorylation in thrombin-induced serotonin release, permeabilized platelets were used. However, it was necessary to establish first the overall response of permeabilized platelets to thrombin stimulation.

1- Effect of permeabilization on thrombin-induced platelet aggregation.

Platelets were permeabilized by incubation for 5 min with 15 μM digitonin at room temperature in K^+-glutamate buffer as described in Materials and Methods. Intact platelets were resuspended in Locke's solution. Aliquots (0.5 ml) of intact or permeabilized platelets suspensions were placed in siliconized glass cuvettes and let to equilibrate for 2 min at 37°C with stirring in a whole blood dual channel aggregometer. Fifty microliters of either vehicle solution or a solution containing thrombin (1U/ml final concentration) were then added to the corresponding preparation and platelet aggregation was monitored by measuring the change in light

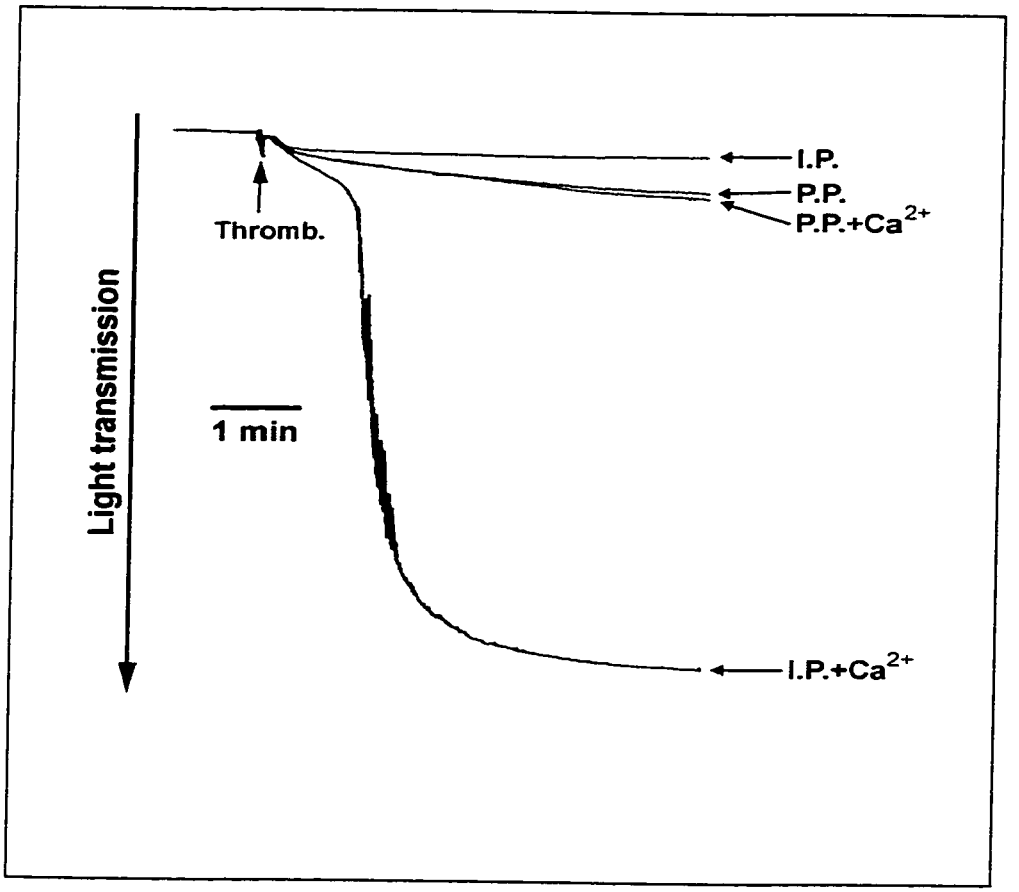

Fig 3.27. ***Effects of permeabilization condition on thrombin-induced platelet aggregation.*** Intact platelets (I.P.) were resuspended in regular Locke's or in Ca^{2+}-free Locke's solution whereas digitonin permeabilized platelets (P.P.) were resuspended in K^{+}-glutamate buffer either in the presence or absence of 10 μM free Ca^{2+}. Five hundred microliter platelet aliquots (7.5×10^{8}platelets/ml) were placed in siliconized glass cuvettes under constant stirring. Platelet preparations were then challenged with 1 U thrombin/ml and aggregation was monitored as described in Materials and Methods. Aggregation was observed only when I.P. were incubated in the presence of Ca^{2+}.

transmission as indicated in Materials and Methods. Under these conditions, thrombin was found to induce aggregation only in intact platelets providing sufficient free Ca^{2+} (2.2 mM) was present in the medium. Neither intact platelets suspended in Ca^{2+}-free Locke's solution, nor permeabilized platelets (in absence or presence of free Ca^{2+}) showed any aggregation in response to thrombin (fig 3.26).

2- Effect of permeabilization on thrombin-induced [^{3}H]5-HT release from platelets.

Platelets were permeabilized by incubation with 15 μM digitonin for 5 minutes at room temperature in K^+-glutamate buffer as described under Materials and Methods. Intact platelets were suspended in Ca^{2+}-free Locke's solution. Aliquots (100 μl) of either intact platelets or digitonin permeabilized platelets were incubated for 3 minutes either with vehicle or thrombin (final concentration 1 U/ml). Intact platelets responded to thrombin stimulation by releasing from 70 to 80% of their total [^{3}H]5-HT content (Fig 3.28). On the other hand, digitonin-permeabilized platelets, although still significantly responded to thrombin stimulation, their release response was reduced by approximately 40% in either high or low chelating agent containing K^+-glutamate buffers (Fig3.28).

K. Effects of MPSD and Ala-MPSD on the [^{3}H]5-HT release from permeabilized platelets induced by thrombin stimulation.

Because MARCKS phosphorylation has close correlation with serotonin

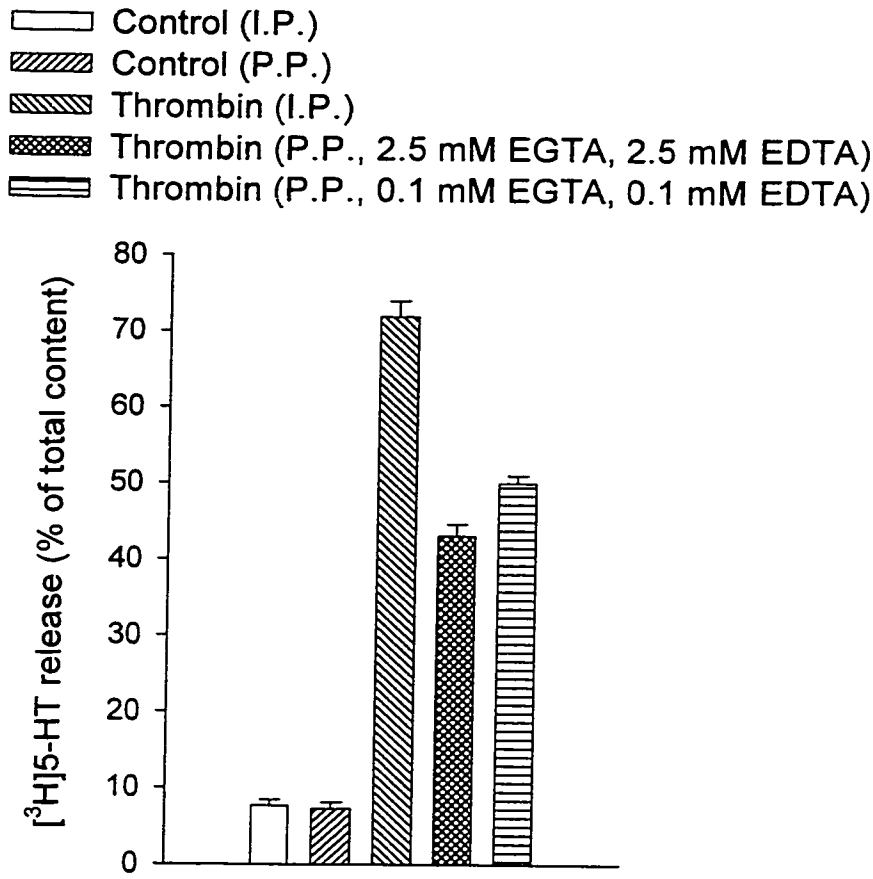

Fig 3.28. Effect of permeabilization on thrombin-induced [^{3}H]5-HT release from platelets. [^{3}H]5-HT-labelled platelets were used. Intact platelets (I.P.) were suspended in Ca^{2+}-free Locke's solution whereas digitonin-permeabilized platelets (P.P.) were suspended in K^+-glutamate buffer containing different concentrations of EGTA and EDTA. Platelets were then incubated in the same buffers for 3 minutes with either vehicle or thrombin (1U/ml). At the end of the incubation period, [^{3}H]5-HT was measured in the medium and in platelets. [^{3}H]5-HT outputs were expressed as percentage of total content. Each bar represent the mean ± SEM of results obtained from 8 different preparations.

release from intact platelets stimulated by thrombin, we used the digitonin-permeabilized platelets preparation to further test whether or not MARCKS phosphorylation is involved in thrombin-induced [^{3}H]5-HT release.

Digitonin-permeabilized platelets suspended in K^+-glutamate buffer (pCa<9) were stimulated with 1 U thrombin/ml for 3 min in absence or presence of 10 μM of either MPSD or Ala-MPSD. As indicated above, digitonin-permeabilization of platelets caused approximately a 40% reduction in the platelet release response to thrombin. As expected, MPSD produced a significant ($P \leq 0.01$, $n = 8$) inhibition of thrombin-induced [^{3}H]5-HT release form permeabilized platelets whereas Ala-MPSD was without effect (Fig 3.29). This would suggest that the PKC-MARCKS pathway mediates part of thrombin-induced release reaction in platelets, since the percentage of MPSD-induced inhibition of thrombin-evoked serotonin release is small (30%) when compared to the inhibition observed with the peptide on PMA-evoked serotonin release (70%).

L. Effects of MPSD and Ala-MPSD on permeabilized platelet protein phosphorylation induced by thrombin stimulation.

Platelets previously labelled with [^{32}P]Pi were incubated for 3 min with thrombin at a concentration of 1U/ml in the absence or presence of 10 μM of either MPSD or Ala-MPSD. This was followed by separation of heat stable proteins by SDS-PAGE as described in Materials and Methods. A representative autoradiograph

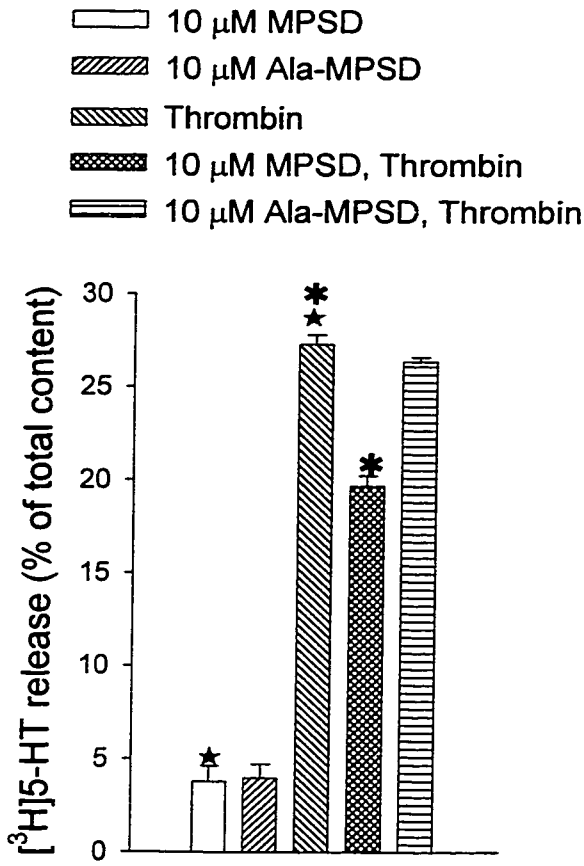

Fig 3.29. Effects of peptides MPSD and Ala-MPSD on thrombin-induced [^{3}H]5-HT release from permeabilized platelets. [^{3}H]5-HT labelled patelets were permeabilized for 5 min with 15 μM digitonin in K^+-glutamate buffer in absence or presence of 10 μM of either MPSD peptide or Ala-MPSD peptide. Platelets were recovered by centrifugation and resuspended in same medium for 3 min in absence or presence of 1 U thrombin per ml. At the end of this stimulation period, [^{3}H]5-HT content was measured in the medium and in platelets as described in Materials and Methods. [^{3}H]5-HT outputs were expressed as percentage of total content. Each bar represent mean±SEM obtained from 8 different preparations (★: $P \leq 0.01$, $n = 8$; ✱: $P \leq 0.01$, $n = 8$).

of four different experiments and its corresponding scannings are depicted in Fig 3.30. When heat-stable protein extracts were analysed, it was observed that stimulation of platelets by thrombin significantly ($P \leq 0.01$, $n = 4$) increased the phosphorylation of both MARCKS and myosin light chain (MLC) as well as other unidentified heat-stable poly-peptides (open triangles, Fig 3.30). In the presence of 10 μM MPSD, there was a significant ($P \leq 0.01$, $n = 4$) inhibition of thrombin-induced phosphorylation of MARCKS (Fig 3.30, lane 4). However, there was no inhibition of thrombin-induced phosphorylation of MLC (Fig 3.30, lane 4). Here again the MPSD peptide was phosphorylated in response to thrombin stimulation (asterisk in Fig 3.30). Furthermore, when 10 μM Ala-MPSD was present in the medium, there was no inhibition of thrombin-induced MARCKS phosphorylation (Fig 3.30, lane 3). The increase in MLC phosphorylation was not affected and, as expected, Ala-MPSD was not phosphorylated (Fig 3.30, lane 3). In another set of experiments, proteins present in unheated platelet extracts were separated by SDS-PAGE. The autoradiography and scanning of one such gel is shown in Fig 3.31. The significant ($P \leq 0.01$, $n = 4$) increase in the phosphorylation of pleckstrin observed in the presence of thrombin was not modified in the presence of either MPSD or Ala-MPSD at concentrations which MPSD inhibits MARCKS phosphorylation and serotonin release (Fig 3.31). Here again the phosphorylation of MLC was not affected by the peptides and phosphorylation of MPSD was observed (asterisk in Fig 3.31).

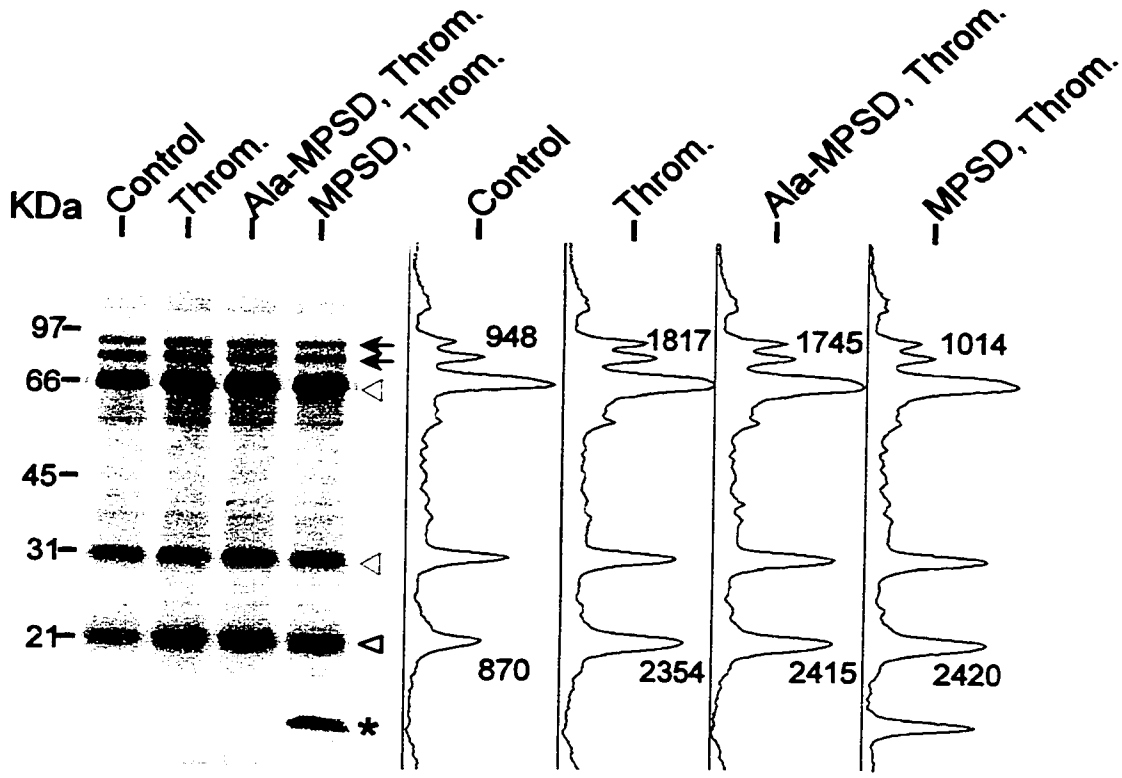

Fig *3.30.Effects of MPSD and Ala-MPSD on the phosphorylation of MARCKS induced by thrombin stimulation.* Platlelets were labelled with [^{32}P]Pi and permeabilized with digitonin in the absence or presence of 10 μM of either Ala-MPSD (lane 3) or MPSD (lane 4) and subsequently stimulated with thrombin as described in the legend to Fig 3.29. At the end of the stimulation periods, heat-stable platelet extracts were prepared and their proteins were separated by SDS-PAGE as indicated in Materials and Methods. Proteins were then electrotransferred to nitrocellulose membranes; these were exposed to Hyperfilm™ and the autoradiographies thus obtained were scanned as indicated in Materials and Methods. The figure shows the autoradiography of an experiment carried out to test the effect of MPSD and Ala-MPSD on protein phosphorylation. Double arrows show the position of MARCKS whereas single arrow heads indicate the position of myosin light chain (MLC). Open triangles indicate the position of other unidentifed heat-stable proteins. The asterisk indicates the position of phosphorylated MPSD.On the right-hand side in both panels, scannings of the autoradiographies are shown. The numbers beside the MARCKS and MLC peaks are arbitrary units obtained from integration of peak areas. Similar results were obtained in 3 other experiments (see Fig 3.31).

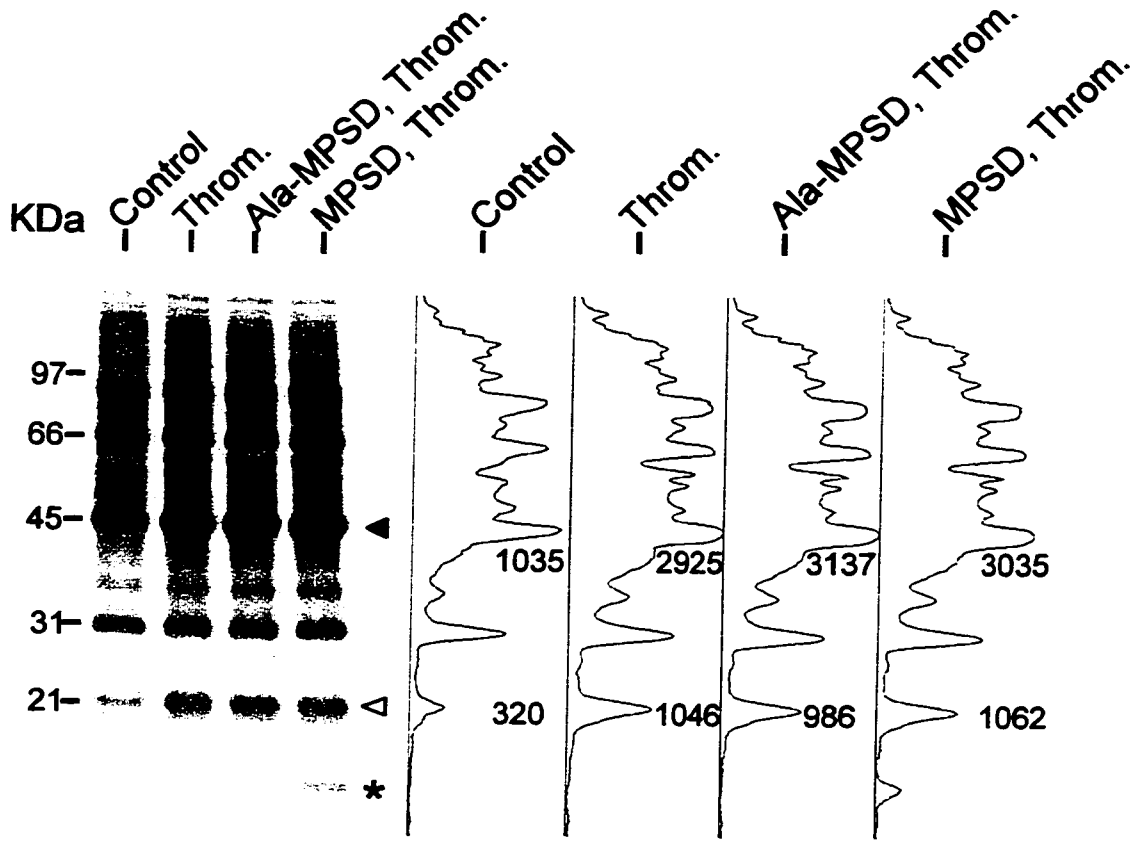

Fig 3.31. Effects of MPSD and Ala-MPSD on the phosphorylation of pleckstrin (p47) and myosin light chain (p20) induced by thrombin stimulation. The protocol for this experiment is similar to that described in legend to fig 3.30, except that total platelet extracts (heat-stable plus heat-sensitive proteins) were prepared. SDS-PAGE, electrotransferring, autoradiography, scanning and integration of peak areas (arbitrary units) were performed as indicated in the legend to fig 3.14 and in Materials and Methods. The close and open arrowheads indicate the positions of pleckstrin and myosin light chain (MLC), respectively. The asterisk shows the position of phosphorylated MPSD. Similar results were obtained in 4 other experiments.

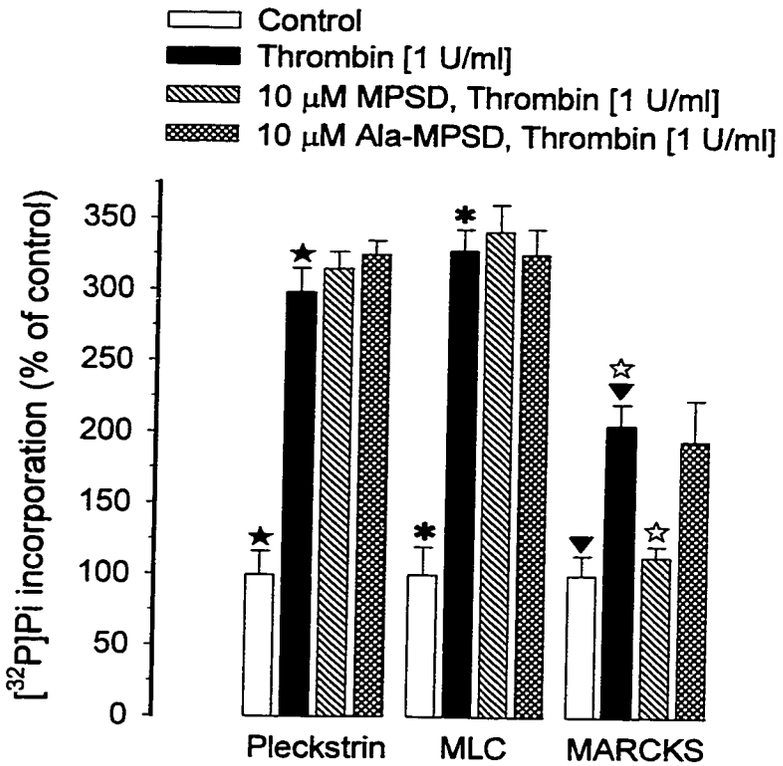

Fig 3.32. ***Cumulative data on the effect of MPSD and Ala-MPSD on thrombin-induced protein phosphorylation in permeabilized platelets..*** Experiments were performed as described in legend to Fig 3.31 for pleckstrin and MLC and as indicated in legend to Fig 3.30 for MARCKS. Bars represent mean ± SEM of [^{32}P]Pi incorporation, expressed as percentage of control (absence of thrombin), obtained from 4 different experiments for each condition tested (★: $P \leq 0.01$, $n = 4$; ✱: $P \leq 0.01$, $n = 4$; ▼: $P \leq 0.01$, $n = 4$; ☆: $P \leq 0.01$, $n = 4$).

Figure 3.32 shows cumulative data from four different phosphorylation experiments on permeabilized platelets. The levels of protein phosphorylations under each condition were determined by densitometric analysis of the autoradiographs and expressed in arbitrary units as percentage of control. The results clearly indicate that only the phosphorylation of MARCKS was affected by MPSD. Differences among groups were found to be significant ($P \leq 0.01$, $n = 4$) using unpaired Student t-test.

Chapter (IV)
Discussion

Upon activation, platelets undergo dramatic ultrastructural changes accompanied by the rearrangement of cytoskeleton components (Carroll at al, 1982; Debus et al, 1981; Cox, 1984; Siess, 1989) and the translocation of several proteins from cytosol to cytoskeleton (Bertagnolli and Beckerle; 1994, Tuszynski et al, 1982; Hynes, 1987). Actin polymerization-depolymerization cycles take place in different areas during platelet activation (Carroll at al, 1982). Long bundles of actin filaments radiating from the platelet centre have been observed in activated platelets (Hartwig et al, 1998). On the other hand, during platelet activation actin filaments together with myosin and other cytoskeleton associated proteins (e.g., spectrin, actin binding protein and membrane associated glycoproteins) form the contractile force that mediates platelets aggregation and retraction of the externally bound platelet clot (Hartwig, 1998). Previous work has shown the presence in platelets of gelsolin (Lind et al, 1982; Lind et al, 1987) and scinderin (Rodriguez Del Castillo et al, 1992), two Ca^{2+}-dependent F-actin severing proteins that control actin network dynamics. It has been suggested that during platelet aggregation, actin polymerizes and the content of the secretory granules is released to the cell exterior (Sherry, 1976; Siess, 1989; Carroll et al, 1982; Hartwig, 1998). Opposite to this view are the results from experiments from our laboratory showing that recombinant scinderin potentiates Ca^{2+}-induced release of serotonin from permeabilized platelets (Marcu et al, 1996; Marcu et al, 1998), This work suggests that, similar to what is observed in other secretory systems (i.e., chromaffin cell, Vitale et al, 1991; Vitale et al, 1995), it is not actin

polymerization, but actin disassembly, perhaps at a specific site, that is required for platelet secretion (Marcu et al, 1996; Marcu et al, 1998). Moreover, it has been shown that treatment of platelets with cytochalasin E, an inhibitor of actin assembly, decreased actin polymerization and inhibited platelet aggregation without affecting granular secretion (Lefebre et al, 1993). Moreover, cytochalasin D caused instability of platelets aggregates (May et al, 1998).

A. Characterization of the permeabilized platelet preparation.

The aforementioned evidence suggests that during platelet activation an enormous increase in actin assembly which mediates other responses (shape change, formation of pseudopodia and lamellae, aggregation and clot retraction) could mask the highly localized F-actin disassembly which mediates platelet secretory vesicle secretion. Therefore, in order to study this phenomenon, the two events (i.e., aggregation and secretion) responsible for the actin cytoskeleton changes must be dissected. In other words, the mechanism(s) of platelet secretion should be studied in a condition of complete absence of platelet aggregation. Evidence has been provided that aggregated platelets show more cytoskeletal changes and protein phosphorylation than non-aggregated platelets (Fox, 1983; Hartwig, 1998). On the other hand, outside-in signals induced by fibrinogen receptor cross-linking result in elevation in cytosolic Ca^{2+} and induction of tyrosine phosphorylation of many platelet proteins (Law et al,

1999). These observations suggest that during aggregation, platelets are subjected to activation of further intracellular signal pathways. Other investigators have used the approach of keeping platelets without stirring to avoid platelet aggregation (Nishikawa et al, 1980; Hashimoto et al, 1997). In our case, we have designed experiments which allowed us to use peptides as tools to investigate the role of PKC in platelet secretion. Therefore, free access to the platelet cytosol was a necessary requirement. Consequently, we have made use of the digitonin-permeabilized platelet system (Marcu et al, 1996) to address the role of actin cytoskeleton during platelet secretion.

Our results show that stirred digitonin-permeabilized platelets do not aggregate under the conditions we used to study secretion (Fig 3.1 and Fig 3.27) but they still secrete in response to different stimuli (e.g., Ca^{2+}, PMA and thrombin, Fig 3.3, Fig 3.10 and Fig 3.28). This preparation provided us with an excellent model to study the molecular mechanism(s) of platelet secretion in complete absence of platelet aggregation. The results show that the degree of permeabilization obtained with digitonin is quite high, as assessed by the uptake of rhodamine-phalloidin (a probe for filamentous actin) by digitonin-treated platelets. In spite of this, the leakage into the medium of proteins dealt with in this study (i.e., PKC, Pleckstrin and MARCKS) was found to be low, between 2 and 28% (Fig 3.5). Nevertheless, a good secretory response was observed up to 15 minutes of permeabilization (Fig 3.4). Moreover, the

leakage of radioactive serotonin during digitonin permeabilization was not significant and only a small amount of [^{3}H]5-HT was found to escape from platelets during permeabilization (Fig 3.2). As in previous experiments on platelet secretion (Marcu et al, 1996), a forty-five-second stimulation was used in most experiments with this preparation, a period showing a significant and the highest rate of secretion (Fig 3.3). Furthermore, it has been shown in another well characterized system, the chromaffin cell, that secretion during the first 45-60 seconds of stimulation corresponds to release from the so-called "release-ready vesicle pool" (Vitale et al, 1995). Although this secretory vesicle pool has yet to be demonstrated in platelets, the secretory behaviour of platelet preparations, especially in the presence of recombinant scinderin (Marcu et al, 1996), suggests that in platelets, as in other secretory systems, a pool of release-ready vesicles may be involved in the initial phase of fast release.

In conclusion, digitonin (at concentration of 15 μM) can induce permeabilization in more than 90% of the platelets suspended in the appropriate buffer (e.g., K^+-glutamate buffer), and the pores created in the cell membrane are large enough to introduce 25 amino acid peptides into the cell interior (Fig 3.17). Furthermore, digitonin-permeabilized platelets maintain their cellular integrity and sensitivity to stimuli for sufficient periods of time. We used this system successfully to investigate the role of protein kinase C (PKC) and its substrates in platelet secretion.

B. Identification of the MARCKS protein in platelets.

Although MARCKS is expressed in wide variety of tissue, with highest amount present in the brain, its existence in platelets has not been previously demonstrated. First discovered in isolated brain synaptosomes (Wu et al, 1982), MARCKS has been shown to be present in a variety of cells such as fibroblasts (Blackshear et al, 1986), macrophages (Allen and Aderem, 1995), neutrophils (Thelen et al, 1990), endothelial cells (Jacobson et al, 1992; Zhao and Davis, 1996), cardiac myocytes (McGill et al, 1997), melanocytes (Brooks et al, 1996), astrocytes (Smith et al, 1996), mesangial cells (Studer et al, 1995), follicular granulosa cells (Stormshak et al, 1995), MEG-01 cell line (a cell line derived from a megakaryocyte leaukemia) (Nagata et al, 1996) and others. The present experiments provide the first demonstration of the presence of MARCKS in platelets. Immunostaining of fixed human platelets using monoclonal antibody against the C-terminal domain of MARCKS shows a positive strong reaction indicating the presence of the antigen. A non-specific mouse IgG antibody was used as a negative control to eliminate the possibility that the immunoreactivity was due to non-specific binding of the MARCKS antibody. At the same time, Anti-CD41a (fibrinogen receptor) antibody was used as a positive control to identify platelets. Furthermore, immunoblotting of platelet proteins separated by SDS-PAGE using the same antibody revealed a double

band of electrophoretic mobility of about 86 kDa, which co-migrate with a band representing MARCKS in cell lines (SH-SY5Y human neuroblastoma cell line and MEG-01) known to express high amount of MARCKS. The antibody recognized a band which was heat-stable, a property of MARCKS protein, and was phosphorylated upon activation of PKC as indicated by a shift in MARCKS band in the autoradiograph of the immunoblot (Fig 5). We used three different antibodies raised against different domains of MARCKS: a monoclonal antibody against the C-terminus, a polyclonal antibody against a peptide mapping the C-terminal domain and a polyclonal antibody against a peptide mapping the N-terminal domain of MARCKS. All three antibodies recognized, in platelet extracts, the same band at approximately 86 kDa. Thus, indicating the presence of MARCKS in human platelets.

Due to difficulty in obtaining a pure preparation of MARCKS, we followed an approach to determine the relative abundance of MARCKS in platelets by using an extract prepared from MEG-01 cells as standard (Fig 3.8). This leaukemia cell line has been shown to contain MARCKS (Nagata et al, 1996), and it originates from megakaryocytes, the platelets progenitors (Ogura et al, 1985). The results obtained in these experiments indicated that the concentration of MARCKS in platelets was 68±3% (n=4) of that in Meg-01 cells.

C. Role of MARCKS in platelet secretion induced by PKC activation.

Two decades of intensive work has established strong evidence of the pivotal

role of protein kinase C (PKC) in cellular signalling systems. This serine/threonine phosphotransferase family is composed of twelve isozymes ubiquitously expressed in every single type of eukaryotic cells (Azzi et al, 1991; Lodewijk et al, 1994; Nishizuha, 1995; Blobe, 1996; Quest, 1996; Jaken, 1996; Majewski et al, 1997; Liu and Heckman, 1998; Mellor and Parker, 1998; Keenan and Kelleher, 1998; Vaughan et al, 1999). PKC activation is thought to mediate a multitude of cellular processes including cell motility, cell differentiation and exocytosis, however, the downstream events that lead to the final response are poorly understood. One such mediator of PKC evoked events is the MARCKS protein whose phosphorylation is considered to be a marker for the activation of PKC pathway in many cell types(Wu et al, 1982; Rodriguez-Pena and Rozengurt, 1986; Stumpo et al, 1989; Graff et al, 1989; Seykora et al, 1991; Brooks et al, 1991; Erusalimsky et al, 1991; Harland et al, 1991; Sakai et al, 1992).

Previous work with other secretory tissues has demonstrated that the release of hormones or neurotransmitters is accompanied by an increase in the phosphorylation of several proteins which are substrates of PKC (Wu et al, 1982; Wang et al, 1989; Calle et al, 1992; Robinson, 1992; Coffey et al, 1994; Liu et al, 1994; Goodall et al, 1997). Although these experiments show some degree of correlation between an increase in MARCKS phosphorylation and hormone or neurotransmitter release, they did not provide a cause-effect relationship between the two events (Wu et al, 1982; Wang et al, 1989; Calle et al, 1992; Coffey et al, 1994;

Liu et al, 1994; Goodall et al, 1997). In the present experiments, using peptide MPSD (with a sequence corresponding to the phosphorylation site domain of MARCKS) we have provided evidence of a direct relationship between MARCKS phosphorylation and serotonin release in response to PKC activation. When MPSD was present in the incubation media both platelet MARCKS phosphorylation and serotonin release were blocked, whereas when an Ala-MPSD (a peptide in which the four PKC substrate serine residues were substituted by alanine residues) was in the media no such a block was observed suggesting the involvement of the serine residues in the effect of MPSD. MPSD not only inhibited these two PKC-dependent responses, but it was also phosphorylated in the process, suggesting that PKC activity remained intact when the peptide was present. The observations that the increase in pleckstrin phosphorylation (also a major substrate of PKC) was not affected in the presence of MPSD also indicated that PKC was active. An additional proof of the selectivity of MPSD inhibition for MARCKS phosphorylation was that other unidentified heat-stable proteins (i.e., p25, p31, p50 and p66) were further phosphorylated in response to PMA and that these increased phosphorylations were not modified in the presence of MPSD (Fig 3.13, open triangles). Moreover, the fact that increases in the phosphorylation of pleckstrin and myosin-light chain (MLC) were observed during MPSD-induced inhibition of MARCKS phosphorylation and serotonin release suggested that either these proteins are unrelated to the transduction pathway in which MARCKS is involved or their involvement in serotonin release is upstream of

MARCKS in the cascade of events leading to exocytosis. In this case, one should consider the possibility that a threshold concentration of phosphorylated pleckstrin is required for this protein to be involved in secretion and that this threshold is only reached at 100 nM PMA. One possible explanation is that MLC phosphorylation is involved in platelet-shape change and/or aggregation (Nishikawa et al, 1980; Ikebe and Reardon, 1990), steps that cannot be studied separately from secretion in intact (non-permeabilized) platelets. Similarly, pleckstrin phosphorylation could play a role in platelet-shape change and aggregation. Furthermore, there was also a dissociation between the PMA concentration-dependent curves of the two protein phosphorylations with the phosphorylation curve for MARCKS being almost identical to the serotonin release curve. The suggestion that pleckstrin is involved in secretion in response to PKC activation comes from a large number of publications, all showing increases or decreases in both pleckstrin phosphorylation and platelet release reaction during stimulation or inhibition of PKC activity (Brooks et al, 1990; Hashimoto et al, 1994; Dalla Via et al, 1996; Kamiguti et al, 1997; Rotondo et al, 1997). However, in these publications there has been no cause-effect demonstration or an agonist-concentration dependency study between these two PKC responses as provided here for MARCKS phosphorylation and serotonin release. An alternative explanation to the different phosphorylation patterns observed with pleckstrin and MARCKS might be that different PKC isozymes are involved in the phosphorylation of the two substrates. If this were the case, the same PKC isozyme should be involved in both

the phosphorylation of MARCKS and the release of serotonin. Such isozyme would have more affinity to the MPSD peptide than other isozymes, which explains the specificity of the peptide in blocking MARCKS phosphorylation without affecting the phosphorylation of other PKC substrates. Indeed, a peptide similar to the MPSD peptide was found to have different kinetic for PKC isozymes with higher V_{max} than the full length MARCKS (Fujis et al, 1994). This was attributed to the fact that a peptide is easier to dissociate from the C kinase because of its missing additional binding sites. Furthermore, it is probable that the ability of the peptide to bind to the membrane (Arbuzora et al, 1998) will cause it to concentrate in the vicinity of PKC-MARCKS reaction site. This could play an important role in the MPSD selective effect. Interestingly, it has been found that not all PKC isozymes are able to phosphorylate MARCKS *in vivo* (Uberall et al, 1997) and *in vitro* (Herget et al, 1995), and the coexpression of MARCKS with different individual PKC isozymes revealed variability in their affinity to phosphorylate the protein (Cabell et al, 1996). Moreover, it is well known that PKC subfamilies (i.e., classical, novel and atypical) vary in their activation requirements and cellular localizations. Platelets have been shown to contain, at least, nine different PKC isoforms including PKCα, βI, βII, γ, δ, ε, ζ, η and θ (Crabos et al, 1991; Baldassare et al, 1992; Wang et al, 1993; Khan et al, 1993). Some of these isoforms have different time-courses and concentration dependances for translocation upon stimulation of platelets with either PMA (Crabos et al, 1991) or thrombin (Baldassare et al, 1992). It should also be mentioned that

substrate specificity of PKC isozymes has been suggested (Hug and Sarre, 1993), but the lack of biochemical evidence and poor understanding of the downstream components of the pathway make it difficult to draw proper conclusion at the present time. Likewise, differential expression and localization of PKC isozymes in different cell types does not seem to be mere functional redundancy but rather functional specificity. It is also likely that PKC anchoring proteins, such as RACKs (Receptor for Activated C-Kinase), play a role in PKC isozymes specificity as they might bind specifically to certain isozymes and bring it in close proximity to particular substrate and away from others. Interestingly, evidence is emerging that RACKs are isozyme specific anchoring proteins that localize each PKC to different cellular compartment (Mochly-Rosen and Gordon, 1998). Taken all this together, it is quite possible that each isozyme member of the PKC family (i.e., PKC isozymes) has a distinct role in the cellular signalling system in platelets. In other words, one PKC isozyme may mediate platelet secretion through phosphorylation of MARCKS and reorganization of the platelet cortical cytoskeleton, whereas, other isozymes are involved in platelet shape change and/or aggregation through other PKC substrates. However, clear demonstration of such specific "sub-pathways" is yet to be provided.

The possibility that the observed inhibitory effect of MPSD on secretion was due to displacement and/or inhibition of CaM and PIP_2 effects (MaIlroy et al, 1991; Glaser et al, 1996), was ruled out in experiments performed in the presence of these molecules. CaM at concentration of 10 μM and PIP_2 in concentrations of up to 100

μM were ineffective in reversing the inhibitory effect of MPSD on PMA-induced [^{3}H]5-HT release (Fig 3.12). Moreover, PIP_2 at the same concentration that was used here showed to be a powerful inhibitor of the potentiation by recombinant scinderin of serotonin release induced by calcium (Marcu et al, 1996).

D. Role of MARCKS in platelet secretion induced by thrombin stimulation.

Thrombin stimulation has been shown to cause increase in MARCKS phosphorylation in bovine pulmonary artery endothelial cells (Zhao et al, 1998) and in human umbilical vein endothelial cells (Jacobson at al, 1992). Such phosphorylation was blocked by H7 and calphostin C, two PKC inhibitors, suggesting the involvement of PKC and MARCKS pathway in the signal transduction linked to activation of thrombin receptor (Jacobson at al, 1992; Zhao et al, 1998). The thrombin receptor is a member of the family of receptors with seven transmembranous domains (heptahelical) which is coupled to effector enzymes through heterotrimeric G proteins (Lapetina, 1990). Platelets express quite high number of these receptors on their surfaces (Harmon and Jamieson, 1986). Activation of the thrombin receptor in platelets leads to the activation of membrane bound phospholipase $C\beta_2$ ($PLC\beta_2$) which in turn hydrolyzes phosphotidylinositol 4,5 bisphosphate (PIP_2) present in the cell membrane to *sn*-1,2 diacylglycerol (DAG) and inositol 1,4,5 triphosphate (IP_3) (Lapetina, 1990; Kroll and Sullivan, 1998). Elevation in cytosolic DAG will lead to

activation of PKC and IP_3 will release calcium from endogenous stores by activation of IP_3 receptors of the endoplasmic reticulum (Kroll and Sullivan, 1998).

Data from our experiments revealed that stimulation of intact platelets with thrombin caused an increase in phosphorylation of MARCKS (Fig 3.21 and Fig 3.23), as well as other proteins (pleckstrin and MLC, Fig 3.22 and 3.23), suggesting that MARCKS phosphorylation might mediate some of the thrombin-induced effects in platelets. Furthermore, the effect of thrombin stimulation on MARCKS phosphorylation in intact platelets was concentration-dependent. Here again, the thrombin concentration-effect curve of MARCKS phosphorylation was almost identical to the [^{3}H]5-HT release curve (Fig 3.24). On the contrary, thrombin-evoked pleckstrin phosphorylation curve was shifted to the left indicating a lower EC_{50} and an enhanced sensitivity to thrombin by this protein than for MARCKS phosphorylation and [^{3}H]5-HT release. This dissociation of effects of thrombin suggests that MARCKS phosphorylation might be involved in thrombin induced activation of some PKC isozymes which are responsible for vesicle secretion. Despite the fact that presence of extracellular Ca^{2+} is an absolute requirement for platelet aggregation to occur (Heptinstall, 1976; Scrutton and Egan, 1979; Taylor and Heptinstall, 1998; Levy-Toledano et al, 1982; Font et al, 1992), vesicular secretion from intact platelets is independent on extracellular Ca^{2+} (Rink et al, 1982; Rink et al, 1983; Haslam and Davidson, 1984). Our results are consistent with these previous observations (Fig 3.1, Fig 3.25 and Fig 3.27). In addition, thrombin-induced

phosphorylation of MARCKS, pleckstrin and MLC was also independent on extracellular Ca^{2+} (Fig 3.26). This does not rule out the possibility that one of these proteins is involved in thrombin-induced platelet aggregation, since the Ca^{2+}-dependent step in platelet aggregation might be upstream of activation of platelet cytosolic kinases. However, these results provide evidence that under this condition (i.e., absence of extracellular Ca^{2+}), secretory mechanisms and signal cascades are unaffected (Fig 3.25 and Fig 3.26) whereas platelets are unable to aggregate (fig 3.27). Interestingly enough, it has been shown that stimulation by thrombin of platelets suspended in Ca^{2+}-free media causes a rapid increase in the cytosolic Ca^{2+} concentration (Doni et al, 1994). This effect seems to be mediated through liberation of Ca^{2+} from the intracellular Ca^{2+} stores that are sensitive to thapsigargin, an inhibitor of ATP-energized Ca^{2+}-pumps (Doni et al, 1994).

The present results demonstrate that intact platelets secrete from 75 to 80% of their total contents of [^{3}H]5-HT upon stimulation with 1 U thrombin/ml (Fig 3.25 and Fig 3.28), a response that seems to involve more than one signal transduction pathway. Digitonin permeabilization of platelets causes approximately 40% reduction in the magnitude of thrombin-induced serotonin release (Fig 3.28). This reduction does not seem to be due to chelation of cytosolic Ca^{2+} by the EGTA and EDTA present in the buffer since the use of 25 times less amount of these chelators in the buffer did not show any change in the response (Fig 3.28, column 5). Thus, digitonin-permeabilized platelets still significantly respond to thrombin stimulation with a 7 to

8 fold increase in [^{3}H]5-HT release. Similarly, Lapetina et al (1985) reported that platelet responses to thrombin are greatly reduced by saponin permeabilization. This digitonin-related detergent was found to completely block thrombin-induced platelet protein phosphorylation and reduced thrombin-induced serotonin release at higher concentrations (Lapetina et al, 1985).

As with platelet stimulation by PMA, MPSD (10 μM) showed a significant inhibitory effect on thrombin-induced [^{3}H]5-HT release from permeabilized platelets. Similarly, the same concentration of Ala-MPSD peptide was without an effect and this demonstrates, as in the case of PMA stimulation, the involvement of the four serine residues of MPSD in the effect. It is important to notice that the inhibitory effect of the MPSD on thrombin-induced platelet [^{3}H]5-HT release is low (~30%) compared with the inhibition caused by the peptide on PMA-induced [^{3}H]5-HT release (~70%), in spite of an inhibitory effect of 90% on MARCKS phosphorylation by the peptide (Fig 3.30). This, probably, represents the inhibition by MPSD of the PKC-mediated contribution to the thrombin-evoked release mechanism in platelets. Furthermore, the present results demonstrate the involvement of MARCKS in thrombin-induced platelet activation process. Thrombin-induced Phosphorylation of MARCKS is, probably, mediated through activation of PKC as a result of the increase in cytosolic free-Ca^{2+} and DAG generated by membrane PLC stimulation.

In view of the present results, a question that immediately comes to mind is how MARCKS is involved in platelet secretion? Early experiments done in our lab

with recombinant scinderin in digitonin-permeabilized platelets, suggested that it is not actin polymerizations as earlier thought but, rather, it is F-actin disassembly, perhaps at a specific site, that is important for platelet secretion (Marcu et al, 1996). Previous experiments had clearly demonstrated that recombinant scinderin (a Ca^{2+}-dependent F-actin severing protein) potentiated Ca^{2+}-induced release, an effect that was blocked by peptides with sequences corresponding to either of the two actin-binding sites of scinderin (Marcu et al, 1996; Zhang et al, 1996) suggesting the requirement of F-actin disassembly in the release process. Cortical actin disassembly required for hormone and neurotransmitter release has been demonstrated in other secretory tissues such as chromaffin cells and laptotropes (Vitale et al, 1991; Vitale et al, 1995; Zhang et al, 1996). Moreover, it has been shown that treatment with cytochalasin E decreased actin polymerization and inhibited platelet aggregation without affecting granular secretion (Lefebre et al, 1993). Work on chromaffin cells has also demonstrated cortical F-actin disassembly and/or filament rearrangement during PKC activation by phorbol esters (Vitale et al, 1995). In these studies an increase in the initial rate of noradrenaline release was observed and demonstrated to be due to an increase in the number of secretory vesicles at the release sites (Vitale et al, 1995). Additional work from our laboratory on digitonin-permeabilized chromaffin cells has demonstrated that the same MPSD peptide used in the present studies blocked cortical F-actin disassembly and/or filament rearrangement in response to activation of PKC by PMA (Rosé et al, 1997). All these observations

seem to suggest that filament disassembly or rearrangement might be involved in the secretory response to PMA. This idea agrees with the results presented here with MARCKS phosphorylation and its inhibition by MPSD. MARCKS is an actin filament binding protein which cross-links actin filaments in some cell types (Rosen et al, 1990). Phosphorylation of MARCKS by PKC decreases its affinity for F-actin and phospho-MARCKS can not cross-link actin filaments (Hartwig et al, 1992), a property reserved to dephospho-MARCKS (Hartwig et al, 1992). Therefore, it is possible that PMA activation of platelet PKC increases the phosphorylation of MARCKS, decreases actin filament cross-linking and decreases the density of F-actin networks. This would facilitate, as in other secretory systems (Vitale et al, 1995; Rosé et al, 1997), the movement of secretory vesicles to release sites and increasing serotonin release (Fig 4.1). It has been further suggested that the Ca^{2+}-induced and PMA-evoked platelet release reactions are distinct mechanisms (Sloan and Haslam, 1997). Data from our laboratory (Marcu et al, 1996; Marcu et al, 1998) suggests that, as in chromaffin cell secretion (Vitale et al, 1991; Vitale et al, 1995), Ca^{2+}-induced platelet secretion involves scinderin activation followed by actin disassembly. Secretion dependent on PKC activation has now been shown to involve MARCKS phosphorylation which would rearrange of F-actin networks by decreasing actin cross-linking. These mechanisms should not be mutually exclusive and, in response to different secretagogues (which in some cases produce both PKC stimulation and Ca^{2+} entry or release from intracellular stores) both mechanisms could operate

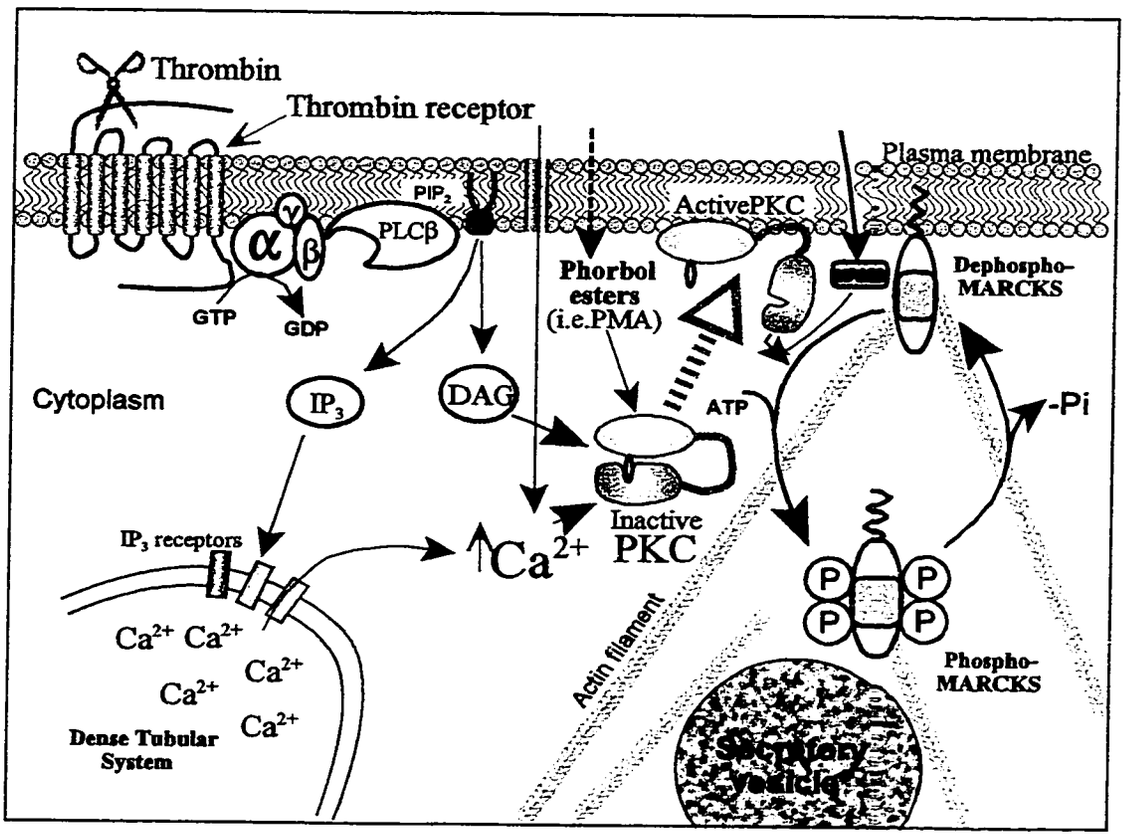

Fig 4.1. Schematic representation of the role of MARCKS in platelet secretion.

simultaneously or sequentially. However, the presence of two parallel or sequential mechanisms involved in platelet release allows different possibilities to modulate platelet secretion.

Conclusion.

The evaluation of our data demonstrates the presence of MARCKS in platelets and suggests a role for this protein in platelet secretion. The similarity of concentration-dependent curves for MARCKS phosphorylation and serotonin release suggests a possible link between PKC activation and the release response in platelets. However, this does not mean that MARCKS mediates all PKC responses in platelets since other major PKC substrates exist in these cells (i.e., pleckstrin). Data presented here provide a cause-effect relationship between MARCKS phosphorylation and platelet secretion. The data also suggests that platelet secretion induced either by direct activation of PKC (by phorbol esters), or receptor mediated signal transduction (i.e., thrombin), involves MARCKS phosphorylation by PKC.

Summary of contributions to original knowledge.

The cellular and molecular mechanism(s) that underlying platelet activation and secretion are not clearly understood. Data presented here contribute new findings to the understanding of the mechanisms involved in platelet secretion. The novel contributions of this study are as follows:

1. A digitonin-permeabilized platelet system was developed. This preparation offers ideal experimental conditions for probing the mechanisms which control platelet secretion in absence of platelet aggregation. Its reproducibility and excellent stability provide a good model to study the molecular mechanisms involved in platelet exocytosis.
2. The first demonstration of the presence in platelets of MARCKS, a **M**yristoylated **A**lanine **R**ich **C** **K**inase **S**ubstrate.
3. The first demonstration of MARCKS phosphorylation upon platelet stimulation either by direct PKC activation (e.g., phorbol esters) or activation through receptor-mediated (e.g., thrombin) signal transduction.
4. A very good correlation between MARCKS phosphorylation and serotonin release in response to both stimuli (i.e., PMA and thrombin) suggest the involvement of MARCKS in the platelet release reaction.

5. Evidence was provided that phosphorylation of pleckstrin and MLC in response to either PMA or thrombin could be observed in the absence of platelet secretion, This, however, does not rule out the possibility that these proteins might have roles upstream in the cascade of events leading to exocytosis.

Chapter (V)
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