FACTORS AFFECTING SECRETION FROM ELECTROPERMEABILIZED HUMAN PLATELETS

By

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ABSTRACT

Comparative studies on the regulation of secretion from dense and α-granules of electropermeabilized human platelets indicated the involvement of three distinct factors, Ca^{2+}, protein kinase C (PKC) and an unidentified GTP-binding protein (G_E). Any two of these factors must be present for marked secretion from dense or α-granules to occur. Thus, Ca^{2+} was not essential to the exocytotic mechanism and the combination of phorbol ester (a PKC activator) and a metabolically-stable GTP analogue (GTP[S]) could produce marked Ca^{2+}-independent secretory response. Ca^{2+} appeared to have a more modulatory role, enhancing both the rate and extent of secretion. Secretion did not correlate with phospholipase C (PLC) activity or with the accumulation of 1,2-diacylglycerol (DAG), both of which required Ca^{2+} and were inhibited by phorbol ester. The Ca^{2+}-independent activation of phospholipase A_2 was shown for the first time in platelets, but moderate to complete inhibition of this enzyme had no effect on secretion or other phospholipase activities. Only the activation of phospholipase D, assayed by the formation of [^{3}H]phosphatidic acid (PA) and [^{3}H]phosphatidyl-ethanol (PEt), in the absence and presence of ethanol, respectively, correlated with secretion under all conditions tested. BAPTA and analogues, known inhibitors of Ca^{2+}-independent secretion in other permeabilized cells, caused

parallel dose-dependent inhibitions of PLD activity and secretion. PKC activity, detected by the phosphorylation of its major endogenous substrate, pleckstrin, was enhanced by GTP[S], apparently by stimulating the formation of PA, as well as of DAG in the presence of Ca^{2+}. An optimal dose of the protein kinase inhibitor staurosporine could not block secretion produced by GTP[S] in the presence of Ca^{2+}, suggesting an additional role for PLD activation, independent of protein phosphorylation. However, although correlations between PLD activity and secretion were also seen in intact platelets, this enzyme is known to account for only 10 - 20% of the total PA formed in intact thrombin-stimulated platelets., This PA may be different from that formed via the PLC - DAG kinase pathway and therefore the nature and subcellular localization of this PA will have to be investigated in order to establish whether PLD has a major role in secretion. The possible existence of an alternate GTP[S]-stimulated effector protein cannot be excluded.

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For Susie,

who made it all the more bearable and worthwhile

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AA	arachidonic acid
ADP	adenosine 5'-diphosphate
ATP	adenosine 5'-triphosphate
BAPTA	1,2-bis(2-aminophenoxy)ethane-N,N,N',N'-tetraacetic acid
BSA	bovine serum albumin
$[Ca^{2+}_i]$	internal free calcium ion concentration
$[Ca^{2+}_{free}]$	free calcium ion concentration
CAL	calyculin A
cAMP	adenosine cyclic 3',5'-monophosphate
$cPLA_2$	cytosolic phospholipase A_2
DAG	*sn*-1,2-diacylglycerol
DGK	diacylglycerol kinase
EDTA	ethylenediamine tetraacetic acid
EGTA	ethyleneglycol-bis-(β-aminoethyl ether) N,N'-tetraacetic acid
G protein	guanine nucleotide binding protein
G_E	putative G protein mediating exocytosis
GAP	GTPase activating protein
GDI	GDP dissociation inhibitor
GDP	guanosine 5'-diphosphate
GIP	GTPase inhibiting protein

GRF	GDP releasing factor
GTP	guanosine 5'-triphosphate
GTPγS	guanosine 5'-O-(3-thiotriphosphate)
GTP[S]	guanosine 5'-O-(3-thiotriphosphate)
5-HT	serotonin
IP	inositol monophosphate
IP_2	inositol 1,4-bisphosphate
IP_3	inositol 1,4,5-trisphosphate
LPA	lysophosphatidic acid
LPC	lysophosphatidylcholine
LPE	lysophosphatidylethanolamine
LPS	lysophosphatidylserine
M_r	molecular weight
MLC	myosin light chain
NSF	N-ethylmaleimide-sensitive factor
OAG	*sn*-1-oleoyl-2-acetyl-glycerol
OKA	okadaic acid
PA	phosphatidic acid
PAF	platelet activating factor
PC-PLC	phosphatidylcholine-specific phospholipase C
PKC	protein kinase C
PGH_2	prostaglandin H_2
PC	phosphatidylcholine
PE	phosphatidylethanolamine
PI	phosphatidylinositol
PIP	phosphatidylinositol 4-phosphate
PIP_2	phosphatidylinositol 4,5-bisphosphate

PI-PLC	phosphoinositide-specific phospholipase C
PLA_2	phospholipase A_2
PLC	phospholipase C
PLD	phospholipase D
PMA	phorbol 12-myristate 13-acetate
PP	protein phosphatase
PS	phosphatidylserine
$sPLA_2$	secretory phospholipase A_2
TXA_2	thromboxane A_2
VWF	von Willebrands factor

This is to officially acknowledge the contributions of Monica Davidson in the assays of [^{3}H]inositol phosphate and [^{3}H]DAG formation cited in Papers 1 (Table 1) and 2 (Table 3), respectively.

INTRODUCTION

1. Platelet Structure and Function

Although often referred to as cells, blood platelets are actually anucleate discoid cell fragments, about 3 μm in diameter, derived from megakaryocytes (Shattil and Bennett, 1980). Although lacking any significant capacity for protein synthesis, platelets otherwise appear to have the major components common to cells proper. An extensive internal membrane structure, the dense tubular system, is involved in the sequestration and stimulus-coupled release of Ca^{2+} (reviewed by Siess, 1989; Rink and Sage, 1990). In addition to mitochondria, platelets also contain three distinct types of storage organelles that are heterogeneous in content and morphological features. Dense granules contain ADP, ATP, serotonin (5-HT) and calcium whereas lysosomes contain a variety of acid hydrolases. The third and most common type, α-granules, contain growth factors and a variety of proteins that are involved in platelet adhesion (e.g. von Willebrand factor; VWF) and aggregation (e.g. thrombospondin), as well as blood coagulation (e.g. factor V) (reviewed by Zucker and Nachmias, 1985; Kaplan, 1986; Siess, 1989). Extensive plasma membrane invaginations form the surface-connected open canalicular system, which is likely to be the primary pathway for secretion from the platelet (Escolar and White, 1991).

In addition to receptors for a wide range of substances that are known to activate platelets, including thrombin, ADP,

collagen, thromboxane A_2 (TXA_2), PAF, and 5-HT (reviewed by Weiss, 1982; Zucker and Nachmias, 1985; Siess, 1989), a variety of glycoproteins, most of which are in some manner involved with platelet adhesion reactions, are also present in the plasma membrane (reviewed by Bennett, 1990). Many of these glycoproteins mediate the ability of platelets to adhere to subendothelium. These glycoproteins include (i) GPIb/IX, the main receptor for von Willebrand factor (VWF) and adhesion to the subendothelium in flowing blood (DeGroot and Sixma, 1990); (ii) GPIa-IIa, the collagen receptor (Nieuwenhuis et al., 1986); and (iii) GPIIb-IIIa, the primary receptor for adhesion in the case of fibrinogen and fibrin and, as the secondary binding site for VWF, necessary for the spreading of platelets on a surface (Weiss et al., 1986; Hantgan et al., 1990; D'Sousa et al., 1991). Both GPIa-IIa and GPIIb-IIIa are recognized as integrins (Bennett, 1990). Directly beneath the plasma membrane lies the membrane cytoskeleton which is linked to particular plasma membrane glycoproteins (e.g. GPIb) in a manner thought to be important for the maintenance of both plasma membrane stability as well as discoid shape (reviewed by Fox, 1987).

In response to blood vessel damage, platelets play integral roles in both the termination of blood leakage and the sealing of the injury site. Upon contacting materials exposed at a trauma site, platelets undergo a variety of changes that have collectively been termed activation; four general responses have

been identified: shape change, adhesion, aggregation and secretion (reviewed by Weiss, 1982). By adhering to materials at sites of vessel trauma, platelets form a physical barrier upon which other platelets can aggregate; the latter process is stimulated by factors released from platelet granules, some of which are also involved in the recruitment of other cells necessary for vessel-wall repair (reviewed by Zucker and Nachmias, 1985; Kroll and Schafer, 1989; Siess, 1989).

During the first few seconds of the shape change reaction the platelet becomes spherical, pseudopods form, GPIIb-IIIa is expressed, and there is major restructuring of the cytoskeleton including phosphorylation of the myosin light-chain (MLC; Daniel et al., 1984). Myosin and actin begin to accumulate around the granules and the contractile action of these proteins results in the centralization of the granules immediately prior to exocytosis (Painter and Ginsberg, 1984). Intragranular fusion and fusion of the granules with the open canalicular system results in the release of granule constituents. Grette (1962; cited in Weiss, 1982; White, 1984) was the first to demonstrate that the release reaction was in fact true secretion involving only the extrusion of granule constituents, but no cytoplasmic factors. Although released granule constituents serve to establish a stable, irreversible aggregation (reviewed by Zucker and Nachmias, 1985; Kroll and Schafer, 1989; Siess, 1989), secretion can also occur in the absence of either adhesion or aggregation. In summary,

platelets may be regarded as secretory cells capable of specific responses to a variety of agonists. Combined with the ease of isolating homogeneous populations, platelets thus represent a unique system for the study of signal transduction and exocytosis.

1.1. Signal Transduction in the Platelet

A key role for Ca^{2+} in platelet activation was originally inferred by analogy to other secretory and contractile cells in which Ca^{2+} had been established as a principal intracellular mediator in stimulus-response coupling (reviewed by Rink and Sage, 1990). Early studies established that Ca^{2+} ionophores, or strong stimuli such as thrombin, could elicit secretion from platelets in the absence of extracellular Ca^{2+}, suggesting that secretion was triggered by Ca^{2+} released from an internal storage site (Feinman and Detwiler, 1974; White et al., 1974). Indeed, the free Ca^{2+} in the dense tubular system has been estimated at several hundred micromolar (Rink and Sage, 1990). This is not to say that extracellular Ca^{2+} (at millimolar concentrations) is unimportant, and indeed Ca^{2+} influx in response to stimulation is involved in the actions of both thrombin and ADP (Sage and Rink, 1986).

Secretion from platelets can be induced by a variety of compounds including thrombin, collagen, TBX_2, ADP and epinephrine. Thrombin is by far the most potent of the known platelet agonists, causing full activation, and secretion from all three granule

types. The recently cloned thrombin receptor appears to be a prototypic G protein-coupled receptor with one distinctive difference; site-specific cleavage by thrombin yields a new extracellular N-terminus that constitutes a tethered ligand for receptor activation (Vu et al., 1991). Activation of phosphoinositide-specific phospholipase C (PI-PLC; see section 1.3.) by thrombin has been well characterized in intact platelets (Rittenhouse-Simmons, 1979; Agranoff et al., 1983; Lapetina, 1990). In addition, the increase in the level of internal free Ca^{2+} ($[Ca^{2+}_i]$), arising from both internal and external sources (Davies et al., 1989), is thought to be involved in the activation of phospholipase A_2 (PLA_2; see section 1.4.) and the subsequent production of TXA_2. Formation of such arachidonic acid (AA) metabolites is essential to secretion induced by low but not high thrombin concentrations (Rink and Hallam, 1984; Sweatt et al., 1985). The effects of collagen have been reported to include the activation of PI-PLC (Watson et al., 1985) although more recent evidence suggests that PLA_2 activation may be a primary response (Piche and Mahadevappa, 1989; Thomas and Holub, 1991). AA metabolites are known to support aggregation at low agonist concentrations (Pollock et al., 1986). The eicosanoid TXA_2 is a fairly potent platelet agonist that interacts with a specific receptor that has only recently been cloned (Hirata et al., 1991). Platelet activation by stable endoperoxide analogues is associated with the stimulation of phosphoinositide breakdown (Rittenhouse, 1984; Siess et al., 1985), an increase in $[Ca^{2+}i]$ and the release

and metabolism of AA. TXA_2 may function primarily to enhance or even support platelet responses initiated by other agonists (Singh et al., 1983; Rink and Hallam, 1984). The nucleotide ADP is another physiologically important platelet agonist that causes secretion from dense and α-granules, but not from lysosomes; AA metabolites appear to mediate the secondary aggregation and secretion (Zucker and Nachmias, 1985). This agonist can elicit a sufficient influx of external calcium to mediate shape change but not full platelet aggregation (Hallam and Rink, 1985). Aside from this increase in $[Ca^{2+}_i]$, no other signal transduction pathways have been definitively linked to ADP-induced platelet activation (Vickers et al., 1990). Perhaps the most intriguing agonist however is epinephrine which, acting at α_2-adrenergic receptors, can elicit aggregation and the secretion of dense and α-granule constituents in the absence of shape change (Zucker and Nachmias, 1985). These responses are mediated by AA metabolites and released ADP (Siess et al., 1984), indicating that the initial signalling pathway for epinephrine might involve PLA_2. There is no evidence of epinephrine-induced PI-PLC activity (Siess et al., 1984). Epinephrine also potentiates platelet responses caused by submaximal concentrations of the previously mentioned agonists (Thompson et al., 1986), possibly as a result of AA metabolism. Indeed, it has been suggested that the effects of epinephrine are entirely dependent on the presence of other aggregating agents, such as ADP (Figures et al., 1986).

Thus, most platelet agonists bring about transient increases in $[Ca^{2+}_i]$ (reviewed by Zucker and Nachmias, 1985; Kroll and Schafer, 1989; Siess, 1989). These transients can be measured using fluorescent indicators such as fura-2 (reviewed by Haslam, 1987; Rink and Sage, 1990). The basal $[Ca^{2+}_i]$ of platelets (~70-80 nM) has been shown to increase to at least 1 μM within 0.2 - 0.4 seconds of stimulation (reviewed by Rink and Sage, 1990). Although the uniformity of this increase in $[Ca^{2+}_i]$ is still in question, studies in adrenal chromaffin cells indicate that exocytosis requires increased Ca^{2+} over the entire subplasmalemmal area (O'Sullivan et al., 1989). At present it is unknown what local influences a heterogeneous increase in $[Ca^{2+}_i]$ might have in platelets. Interestingly, in line with its recognized role as an inhibitor of platelet function, increased levels of the second messenger cyclic AMP (cAMP) have been found to delay the release of Ca^{2+} from internal stores (Tohmatsu et al., 1989; Rink and Sage, 1990). Whereas agonist-induced elevations of $[Ca^{2+}_i]$ are generally accepted as important for stimulus-response coupling, several platelet responses including secretion, myosin light chain phosphorylation and shape-change can occur without a measurable elevation of $[Ca^{2+}_i]$ (reviewed by Haslam, 1987; Siess, 1989). Most of these responses have been related to protein kinase C (PKC; see section 1.6.), which can be activated at basal $[Ca^{2+}_i]$ by diacylglycerol arising from the agonist-stimulated activation of PLC (Rink et al., 1983; Nishizuka, 1984, 1986; Haslam, 1987). At least two other effector enzymes in platelets, PLA_2 and

phospholipase D (PLD) could yield second messengers capable of activating PKC and/or increasing $[Ca^{2+}_i]$.

Since the original description of agonist-modulated incorporation of phosphate into membrane phospholipid (reviewed by Hokin, 1985), there has been escalating interest in the role of membrane phospholipid turnover in cellular signalling. Primarily this focus has been on the hydrolysis of polyphosphoinositides by PLC. In the platelet, early studies showed that thrombin caused a rapid increase in the amount of 1,2-diacylglycerol (DAG) (Rittenhouse-Simmons, 1979). This transient increase of DAG in the membrane, formed in response to an agonist, primarily arises from phosphatidylinositol 4,5-bisphosphate (PIP_2) and phosphatidylinositol-4-phosphate (PIP), via the activation of PLC (reviewed by Haslam, 1987; Siess, 1989). In addition, agonists that induce platelet aggregation without activating PLC do not stimulate the formation of DAG (reviewed by Siess, 1989).

Activation of PLC liberates both DAG and inositol phosphates from polyphosphoinositides. For its part, DAG appears to be a primary physiological regulator of PKC, although other lipids may also be important (see section 1.6.). The polyphosphoinositides represent only a minor fraction of the overall membrane phospholipids; with PIP and PIP_2 as substrates for PLC, the inositol phosphates formed would be inositol 1,4-bisphosphate (IP_2) and inositol 1,4,5-trisphosphate (IP_3),

respectively. The early formation of IP_3 in response to numerous aggregating agents including thrombin and PAF has been well characterized in intact human platelets (reviewed by Haslam, 1987; Siess, 1989). It is well established that IP_3 can stimulate the release of Ca^{2+} from intracellular stores in many cell types (reviewed by Berridge, 1987). IP_3 has been shown to release Ca^{2+} from platelet vesicles loaded with $^{45}Ca^{2+}$ and from isolated preparations of the dense tubular system (Authi and Crawford, 1985; Brass and Joseph, 1985; Enouf et al., 1987; O'Rourke et al., 1987). The similarity in the amount of Ca^{2+} released by IP_3 or by Ca^{2+} ionophore indicates that the former can empty the store of mobilizable Ca^{2+}; indeed, addition of IP_3 to saponin-permeabilized platelets also results in aggregation and secretion (Israels et al., 1985; O'Rourke et al., 1987; Tohmatsu et al., 1989).

The activation of PLA_2 also occurs during, and may be required for, platelet activation. PLA_2 cleaves phospholipids at the C-2 position to release arachidonic acid (AA) and a lysophospholipid (reviewed by Kroll and Schafer, 1989; Siess, 1989); the DAG-lipase pathway is unlikely to contribute a significant amount of AA compared to that generated by platelet PLA_2 activity (Mahadevappa and Holub, 1986). Although there is mounting evidence that AA may itself serve as a second messenger (see section 1.4.2.), most is quickly converted to a variety of eicosanoids. In platelets, one of the major metabolites is TXA_2,

formed via the conversion of AA by cyclooxygenase and thromboxane synthase. TXA_2 is a potent agonist that activates platelet PI-PLC and thus promotes aggregation when released by activated platelets (reviewed by Kroll and Schafer, 1989; Siess, 1989). Furthermore, AA (and metabolites) may also enhance PKC activity and secretion (see sections 1.4.2. and 1.6.1.).

In addition to PLC and PLA_2, thrombin has also been shown to stimulate a phospholipase D (PLD) activity in intact human platelets (Rubin, 1988; Huang et al., 1991b). The significance of this activity has not been established although some studies suggest that the lipid metabolite, phosphatidic acid (PA), could have second messenger functions (see section 1.5.2.). PA can be further metabolized by a specific PLA_2 (Billah et al., 1981) to yield AA and lysophosphatidic acid (LPA), which may have a role in platelet signal transduction (Benton et al., 1982; Gerrard and Robinson, 1984). Alternatively, PA can be metabolized by phosphatidate phosphohydrolase to produce DAG, and this mechanism has been proposed as the source of late secondary DAG elevations seen in many cells (Billah and Anthes, 1990).

A major obstacle to the study of signal transduction mechanisms has been the general inaccessibility of the intracellular environment to direct manipulation; platelets are no exception. Beyond the use of isolated subcellular components or intracellular probes, selective removal of the plasma membrane

diffusion barrier has proven to be one of the most successful approaches to the study of signal transduction mechanisms. In order to be effective, this technique should necessarily employ agents that are selective for the plasma membrane while preserving normal ultrastructural and biochemical properties. A number of agents, that meet these criteria to varying degrees, have been successfully employed in cell suspensions; included are certain detergents, bacterial cytolysins, viruses, ATP^{4-}, and high voltage electrical discharges (electropermeabilization) (reviewed by Ahnert-Hilger and Gratzl, 1988; Knight et al., 1989; Hersey and Perez, 1990). Specific agents, in combination with appropriate experimental conditions, can provide selectivity in terms of both pore size and stability. In general, Sendai virus, *Staphylococcus* α-toxin and electropermeabilization produce the smallest lesions (~2-4 nm), whereas detergents and streptolysin-O produce pores of macromolecular dimensions that permit exchange of many cytosolic proteins. Although electropermeabilization has been a successful approach to the study of platelet signal transduction mechanisms, it is also the most specialized in terms of equipment required (Knight and Scrutton, 1980, 1986b; Haslam and Davidson, 1984a). Thus, although being the biochemically "cleanest" of the available techniques, platelet electropermeabilization has been routinely used by only a few research groups; a detailed account of relevant findings is presented later (see section 1.9.). Conversely, the detergent saponin has been the most widely utilized agent for platelet permeabilization in studies of signal

transduction. In general, this latter approach has provided evidence consistent with a second messenger role for the PLC product IP_3 in the mobilization of an internal Ca^{2+} store and enhanced the evidence that PLA_2 activation and the subsequent metabolism of AA are important for platelet function (Watson et al., 1986; Nakashima et al., 1987; Tohmatsu et al., 1989; Yang et al., 1992). In addition, this technique has contributed to the confirmation of a role for distinct G proteins in the receptor-mediated activation of both PLC and PLA_2 (Baldassare and Fisher, 1986; Nakashima et al., 1987; Akiba et al., 1989; Krishnamurthi et al, 1989; Murayama et al., 1990).

1.2. GTP-Binding Proteins

Extensive evidence now indicates that many of the receptors involved in signal transduction are coupled to their effector system(s) by GTP-binding proteins (G proteins). In addition to transducing and amplifying the signal inherent to a given receptor-ligand complex, these G proteins introduce a level of specificity to the subsequent response(s) and can integrate multiple signalling pathways.

1.2.1. Receptor - Effector Coupling

To date, the G proteins that have been directly implicated in hormone signalling are all heterotrimers consisting of α, β,

and γ subunits (reviewed by Kaziro et al., 1991; Simon et al., 1991). The function and specificity of each G protein was classically thought to be determined by the α subunit which binds GTP and contains GTPase activity. In the absence of $\beta\gamma$ subunits, activated α subunits have been shown to stimulate specific enzyme activities directly. Although the β and γ subunits were originally thought to be somewhat nonspecific in character because of their interchangeability with a variety of α subunits, they are now recognized to be more selective in their interactions with each other as well as with α subunits (Pronin and Gautam, 1992). The β subunit is now thought to have a role in the specific coupling between a receptor and an effector (Kleuss et al., 1992) and, together with a γ subunit, may act to anchor α subunits to specific receptors (Kurstjens et al., 1991). Although the $\beta\gamma$ subunits may oppose agonist induced effector activation, these subunits have more recently been recognized as mediators in their own right. Studies have shown that, in the presence of free α subunits, $\beta\gamma$ subunits can activate type-II and IV adenylyl cyclases and may inhibit the type-I enzyme (Tang and Gilman, 1991). In addition, $\beta\gamma$ subunits have also been shown to regulate atrial K^+ channels (Neer and Clapham, 1988; Kim et al., 1989), stimulate both PLA_2 (Axelrod et al., 1988) and PLC (Camps et al., 1992), and regulate a plasma membrane Ca^{2+} pump (Lotersztajn et al., 1992).

Agonist activation of effectors involves the sequential

transfer of information from the receptor to the effector(s) via G proteins. Receptor occupation by an agonist causes receptor – G protein coupling; the agonist–receptor complex promotes the otherwise slow GDP/GTP exchange on the α subunit by inducing both the release of GDP and the binding of GTP. This binding is the rate–limiting step in effector activation and causes the dissociation of the receptor – G protein complex (reviewed by Kaziro et al., 1991; Simon et al., 1991). At this stage, the α subunit, with bound $Mg^{2+}GTP$, dissociates from the βγ subunits and both are thus available to interact with specific effector systems (most often enzymes or ion channels). Activation by α subunits is terminated by the intrinsic GTPase activity of the α subunit which then reassociates with βγ subunits. Free βγ subunits can accelerate the rate of α subunit deactivation and slow the dissociation and activation of the heterotrimer by Mg^{2+} and nonhydrolysable GTP analogues. Since a given receptor might interact with several G proteins during the period of exposure to an agonist and since the activated α subunit has a fairly long half–life, this mechanism of receptor – effector coupling can amplify the signal of an agonist more than a thousand–fold (Kaziro et al., 1991; Simon et al., 1991).

1.2.2. Characterization of Heterotrimeric G Proteins

Heterotrimeric G proteins are solubilized from membranes by detergents as monomers of about 100 kDa, but the subunits provide

for a diversity not obvious from this simple isolation; α (39 – 46 kDa), β (35 – 37 kDa) and γ (8 – 10 kDa) (Boege et al., 1991). As discussed above, only the α subunit binds and hydrolyses GTP, and some of these subunits are also the targets for ADP-ribosylation by cholera toxin (G_t and G_s) or pertussis toxin (G_t, G_o, G_i) which have proven to be extemely useful tools for identifying G proteins in membranes and for studying their functions. In addition, fluoroaluminate has been found to mimic the γ-phosphate of GTP and thus activate G proteins even in the GDP-bound state (Bigay et al., 1985). More recently, mastoparan and other small amphiphilic peptides have also been shown to interact with and activate α subunits (Higashijima et al., 1990; Mousli et al., 1990).

Utilizing peptide-directed antibodies and molecular biological approaches, sixteen α subunit genes have so far been identified; alternate splicing accounts for the occurrence of further variations among identified α subunits (reviewed by Iyengar and Birnbaumer, 1990; Boege et al., 1991). This technology has also been used to identify four distinct β subunits and four γ subunits.

1.2.3. Characterization of Low-M_r GTP-Binding Proteins

The members of this rapidly expanding family of G proteins are unique in that they consist of a single GTP-binding polypeptide of 20–30 kDa. At least 50 such mammalian proteins

have now been identified and can be divided into the distinct subfamilies ras, rho, rab, ran and arf, each consisting of numerous subtypes (reviewed by Boege et al., 1991; Chardin, 1991; Kahn et al., 1992; Takai et al., 1992). To date, several low-M_r GTP-binding proteins have been identified in platelets including ras, rap, rho, rac and rab species (Bhullar and Haslam, 1987, 1988; Bhullar et al., 1990; Nagata et al., 1990; Ohmstede et al., 1990; Manning and Brass, 1991). All identified small GTP-binding proteins are isoprenylated, palmitoylated, or both (reviewed by Takai et al., 1992). These proteins are not generally affected by fluoroaluminate (Kahn, 1991) or bacterial toxins with the exceptions of the rho and rac proteins which are ADP-ribosylated by botulinum C3 exoenzyme (reviewed by Aktories and Hall, 1989; Boege et al., 1991; Takai et al., 1992). These small GTP-binding proteins have only a low intrinsic GTPase activity. A group of recently identified regulatory factors control the nature of the guanine nucleotide (GDP or GTP) present on a given low-M_r G protein (reviewed by Macara, 1991; Takai et al., 1992). These regulatory proteins include (i) GDP dissociation inhibitors (GDIs); (ii) GDP releasing factors (GRFs); (iii) GTPase activating proteins (GAPs); and (iv) GTPase inhibiting proteins (GIPs). In principle, the effects of GRFs and GIPs should enhance the functional efficacy of small G proteins, whereas GDIs and GAPs should reduce it. Evidence now indicates that there may be a specific form of each regulatory factor for each particular small GTP-binding protein. Polyphosphoinositides, PA with long-chain

fatty acids and AA (as well as its lipoxygenase metabolites) have proven to be inhibitory to a purified GAP, whereas certain prostaglandins may be activators (Tsai et al., 1989a,b; Macara, 1991; Takai et al., 1992). GIP activity has been shown to be enhanced by PIP, PA, AA and DAG. In addition, GRF activities are inhibited by acidic phospholipids including PI, PIP, PIP_2, PS and PA (Takai et al., 1992). A modification of the regulatory effects of these proteins by phosphorylation has only recently been established (Bauer et al., 1992).

1.2.4. Role of GTP-Binding Proteins In Secretion

As the primary transducers of extracellular information across the plasma membrane, the heterotrimeric G proteins influence secretion through the activation (or inhibition) of various effector proteins that control second messenger pathways; these proteins are in effect the initiators of intracellular responses, set in motion by an agonist-receptor interaction at the cell surface. The control intrinsic to such regulatory mechanisms is further refined by the possible interactions of more than one type of G protein with a given agonist-receptor complex. As outlined throughout this introduction, G protein-mediated effectors include enzymes (e.g. phospholipases) and ion channels, the functions of which are likely to be related, either directly or indirectly, to secretory responses (reviewed by Gomperts and Tatham, 1988; Lochrie and Simon, 1988; Plattner, 1989; Sato, 1989;

Sternweis and Pang, 1990). However, substantial evidence from a variety of cells including neutrophils (Barrowman et al., 1986, 1987) and mast cells (Gomperts et al., 1986) has proven that guanine nucleotides can elicit secretion, independently of mediators such as Ca^{2+} and protein phosphorylation (PKC activity). In the light of this evidence, Gomperts and colleagues have postulated the existence of a GTP-binding protein (designated G_E), distinct from that coupled to PI-PLC, which may be an essential mediator of exocytosis (reviewed in Gomperts, 1990; Tatham and Gomperts, 1991), and suggest that the effector of this G protein is likely to be a protein phosphatase (Churcher et al., 1990b). A correlation between secretion and protein dephosphorylation has been recognized in several cells (reviewed by Gomperts and Tatham, 1988).

In platelets, the activities of adenylyl cyclase, PI-PLC, PLA_2 and PLD are known to be directly regulated by G proteins (Haslam and Davidson, 1984c; Haslam et al., 1985; Silk et al., 1989; Van der Meulen and Haslam, 1990). Except for their stimulation by agonists there is as yet no evidence that the G proteins regulating PLA_2 and PLD are heterotrimeric. Although there is weak evidence that phospholipases A_2 and C may be regulated by low-M_r GTP-binding proteins (Bar-Sagi and Feramisco, 1986; Lapetina, 1990), which could explain the ability of microinjected ras protein to stimulate secretion from mast cells (Bar-Sagi and Gomperts, 1988), the best evidence now available

concerning the possible functions of such G proteins in secretion comes from genetic studies of constitutive secretion in yeast. Small GTP-binding proteins, products of the YPT1 and SEC4 genes of S. cerevisiae, are intimately associated with the regulation of vesicular transport and constitutive secretion in this species (Schmitt et al., 1986; Salminen and Novick, 1987; Goud et al., 1988; Segev et al., 1988; Kaiser and Schekman, 1990). In addition to the SEC4 and YPT1 proteins, which belong to the rab family of small GTP-binding proteins, members of the arf family also mediate secretion through a regulatory function in vesicular traffic (Donaldson et al., 1992). Furthermore, both rho and rac have recently been linked to cytoskeletal regulation (Ridley and Hall, 1992; Ridley et al., 1992), and rac1 and 2 have been identified as activators of NADPH oxidase leading to superoxide production in phagocytic cells (Abo et al., 1991; Knaus et al., 1991). In addition, an N-ethylmaleimide - sensitive factor (NSF; now defined as NEM-Sensitive Fusion protein) identified in a mammalian Golgi preparation (Melancon et al., 1987; Block et al., 1988; Wilson et al., 1989; Rothman and Orci, 1990) has also been implicated in constitutive secretion. This factor is postulated to form part of a multimeric "fusion machine," the final trigger for secretion (Malhotra et al., 1988). This model is somewhat similar to the concept of a fusion pore (see section 1.4.2.) that, based on electrophysiological evidence, mediates the final step in exocytosis from a number of cell types.

That the regulation of membrane transport, targetting and fusion may be a general function of several low-M_r GTP-binding proteins (even in regulated secretion) is also suggested, at least circumstantially, by the association of such proteins with secretory granule membranes (Pfeffer, 1992) from a variety of cells including neurons (Matsuoka and Dolly, 1990), pancreatic cells (Padfield and Jamieson, 1991) and human platelet α-granules (Van der Meulen et al., 1991). A possible cytoskeletal function in the platelet has also been suggested for rap1B (Fischer et al., 1990). Heterotrimeric G proteins have also been identified on secretory granules from neutrophils (Rotrosen et al., 1988), chromaffin cells (Toutant et al., 1987), pancreatic cells (Lambert et al., 1990) and parotid cells (Watson et al., 1992). The neutrophil is a particularly intriguing example, where ~35% of the total G protein identified was associated with the specific granule fraction (Rotrosen et al., 1988); most was redistributed to the plasma membrane following agonist stimulation. This could represent a novel mechanism with which to control certain surface receptors and their activities. Alternatively, the localization in granules could indicate either a more direct role for some heterotrimeric G proteins in secretion, or the presence of trans-granule membrane signalling mechanisms that mediate secretion, in a more classical sense, via the activation of effector enzymes and/or ion channels. There is evidence from the work of Chock and colleagues (Chock et al., 1991; Chock and Schmauder-Chock, 1992) that both PLA_2 and a cyclooxygenase are active in the secretory

granules of mast cells; a guanine nucleotide-stimulated PLA_2 activity has also been reported in pancreatic zymogen granule membranes (Rubin et al., 1991) and a PLD activity localized to the azurophilic granules of neutrophils (Balsinde et al., 1989). Whether these activities can be regulated by heterotrimeric and/or low-M_r G proteins sensitive to cytosolic signals, and how these activites might be relevant to secretion has yet to be determined. Additionally, these granule-associated G proteins might trigger a local enzymatic reaction at the point of membrane-membrane contact to facilitate fusion, perhaps as part of the "machine" or pore thought to mediate secretory fusion. Mechanistic investigations are clearly lacking in this area that would appear rife with possible answers to the secretion/fusion puzzle. The permeabilized platelet would be an excellent model system for such studies.

1.3. Phospholipase C

Phosphoinositide-specific PLC (PI-PLC) cleaves the phosphodiester bond in PI, PIP and PIP_2 to form DAG and the corresponding inositol phosphates (IP, IP_2 and IP_3). These phospholipids, which are predominantly present in the inner leaflet of the plasma membrane, constitute a small (< 10%) labile fraction of the total membrane phospholipid in eukaryotic cells. Specific kinases and phosphomonoesterases that regulate the phosphate complement of the inositol group have been identified

(reviewed by Abdel-Latif, 1986); PI is converted to PIP by a PI kinase, and PIP is subsequently converted to PIP_2 by a distinct PIP kinase (Berridge, 1987). It is now generally recognized that receptor-mediated activation of PI-PLC initially produces DAG and IP_3 (Berridge and Irvine, 1989) though, in the platelet, IP_2 may also be a major metabolite (Culty et al., 1988). The evidence to date indicates that DAG and IP_3 are the principal second messengers responsible for mediating a wide range of physiological events (Nishizuka, 1984, 1986; Berridge and Irvine, 1989). DAG is present in the membrane only transiently, and is rapidly converted to PA by DAG-kinase (see section 1.6.3.).

1.3.1. Characterization of PI-Specific PLC Species

Several PI-PLC isozymes have been isolated from both the cytosolic and particulate fractions of a number of mammalian cell types, including human platelets, and at least five immunologically distinct species have been identified (reviewed by Rhee et al., 1989). More recent studies have revealed that at least 14 mammalian isozymes can be distinguished by cloning, and that these enzymes can be divided into three types: PLC-β (~140 kDa), PLC-γ (~150 kDa) and PLC-δ (~90 kDa), each containing a number of subtypes (reviewed by Rhee et al., 1989; Rhee and Choi, 1992). In general, two regions of homology (designated X and Y) are highly conserved between the three PLC types, while subtypes also share increased sequence identity outside these regions. The

Y region contains an amino acid sequence homologous to known Ca^{2+}-binding domains, suggesting that the Ca^{2+}-dependency of PI-PLC activity may involve the binding of this divalent cation to the enzyme. Intact X and Y regions are known to be required for full hydrolytic activity (Dennis et al., 1991). In addition, whereas all three types of PI-PLC catalyse the hydrolysis of PI, PIP and PIP_2, the latter two lipids are the preferred substrates at physiological Ca^{2+} concentrations, with PLC-β1 showing the highest selectivity for PIP_2, and PLC-γ1 the least (Ryu et al., 1987).

Although the presence of Ca^{2+} is necessary, but not sufficient for PLC activation, there is mounting evidence for the involvement of G proteins in the activation of at least some types of PI-PLC. Activation by GTP or its nonhydrolysable analogues, in several cells including platelets (Haslam and Davidson, 1984a,c; Baldassare and Fisher, 1986; Hrbolich et al., 1987; Culty et al., 1988), suggested a role for G proteins in PLC activation. Stimulation by fluoroaluminate, and in some cases, sensitivity to pertussis toxin, indicated that the transducers were likely to be heterotrimeric G proteins (reviewed by Fain, 1990; Exton et al., 1992; Rhee and Choi, 1992). Recently, concerted work by several groups (reviewed by Rhee and Choi, 1992) has proven that members of the G_q subfamily activate PLC-β isozymes, and that there appears to be specificity in the interactions between different G_q and PLC-β subtypes. Indeed, Berstein et al. (1992) have specifically identified PLC-β1 as a GTPase-activating protein for

its own regulatory protein, $G_{q/11}$. In addition, it has been suggested that PKC phosphorylation of PLC-β1 might alter its interaction with G_q (Ryu et al., 1990). In contrast, PLC-γ appears to be activated by both receptor and nonreceptor protein tyrosine kinases. Furthermore, the activation of PLC-δ, which has not yet been associated with any receptors or transducers, has recently been linked to polyamines and basic proteins (Haber et al., 1991).

Banno et al. (1992) have successfully isolated several PLC isozymes from human platelet cytosol. These enzymes have been identified immunologically as PLC-β, -γ1, -γ2, -δ and two unidentified species. With the identification of G_q in human platelets (Manning and Brass, 1991), the direct involvement of at least PLC-β in platelet signal transduction seems likely. Furthermore, from studies involving rabbit platelet membrane preparations, Haslam et al. (1988) have suggested that PI-PLC activity is membrane-associated, and that cytosolic factors are unlikely to play a major role in platelet signal transduction in this species. Banno et al. (1992) have suggested that PLC isozymes in human platelets could be translocated to the membrane, upon platelet stimulation, in a complex with actin and gelsolin. Considering the differential distribution of these enzymes in mammalian cells, and their distinct individual characteristics, it is likely that these PI-PLC isozymes have different functions and/or mechanisms of activation *in vivo*.

1.3.2. Role of PI-PLC Metabolites in Signal Transduction

In platelets labelled with ^{32}P and/or [^{3}H]AA a number of agonists, including thrombin, TXA_2 and collagen, have been shown to initiate the formation of [^{32}P]PA and [^{3}H]DAG; concurrent decreases in PI (Rittenhouse-Simmons, 1979), PIP and PIP_2 (Billah and Lapetina, 1982), accompanied by increases in IP, IP_2 and IP_3 (Agranoff et al., 1983), have also been observed (reviewed by Haslam, 1987). The degradation of PIP_2 occurs within seconds, independently of increases in [Ca^{2+}_i], and is not induced by Ca^{2+} ionophore (Rittenhouse, 1984), which does promote the later degradation of PI (Wilson et al., 1985). In platelet membrane preparations, the evidence indicates that PIP, which is much more abundant than PIP_2, is also a major substrate of PI-PLC (Hrbolich et al., 1987; Culty et al., 1988). Although different isomers and various "higher" inositol phosphates have been identified (reviewed by Berridge and Irvine, 1989), only IP_3 has been shown to serve as a distinct second messenger molecule, stimulating Ca^{2+} mobilization from components of the endoplasmic reticulum in most cells (Berridge, 1987) and probably from the dense tubular system in platelets (Brass and Joseph, 1985; O'Rourke et al., 1987; Ebbeling et al., 1992). Recent investigations have also suggested that IP_3 and inositol 1,3,4,5-tetrakisphosphate, a product of IP_3 phosphorylation, may facilitate the agonist-induced influx of external Ca^{2+} (Bird et al., 1991; Luckhoff and Clapham, 1992) and that the dephosphorylation product of this compound, inositol

1,3,4-trisphosphate, may facilitate Ca^{2+} release from internal stores during more prolonged responses (Berridge and Irvine, 1989).

The role of DAG as a principal physiological activator of PKC has been well documented (Nishizuka, 1984, 1986; Kikkawa and Nishizuka, 1986), and this information is summarized in relation to PKC (see section 1.7.). Less widely recognized is the ability of DAG to destabilize model membrane systems (Das and Rand, 1984; Epand, 1985), resulting in fusion (Nieva et al., 1989; Siegel et al., 1989). Although these systems are meant to model the behaviour of local sites of DAG within a natural membrane, they represent a reductionist approach and thus lack components and factors that mediate the control and specificity characteristic of fusion *in vivo*. Interestingly however, this ability of DAG to perturb bilayer structure is enhanced at physiologically relevant concentrations of cholesterol (Coorssen and Rand, 1990). Such DAG-induced membrane destabilizations may also potentiate the activities of phospholipases, in particular PLA_2 (Dawson et al., 1984; Buckley, 1985; Kolesnick and Paley, 1987; Burch, 1988) and DAG is also suggested to mediate the translocation of PLA_2 to the membrane (Roldan and Mollinedo, 1991). Whether PLD activity might be similarly affected has yet to be investigated. Direct involvement of DAG in the activation of a sphingomyelinase, the metabolites of which may inhibit PKC and/or stimulate PLD, suggests yet another regulatory role for this lipid (Hannun et al., 1986; Kolesnick and Clegg, 1988; Lavie and Liscovitch, 1990).

In addition, DAG has been identified as one of the lipids that enhances the activity of a regulatory factor for low-M_r GTP-binding proteins (Takai et al., 1992). Thus, DAG may have roles additional to the activation of PKC that should be considered in any investigation of secretion.

1.3.3. Identification and Role of PC-Specific PLC

Besterman et al. (1986) were the first to demonstrate the existence of a PC-specific PLC (PC-PLC) activity in a number of cells although a year previously, Wolf and Gross (1985) reported a similar activity in canine myocardium. In response to a variety of agonists, including hormones, phorbol esters and nonhydrolysable GTP analogues, diverse cell types produce DAG and choline phosphate (reviewed by Billah and Anthes, 1990; Exton, 1990; Dennis et al., 1991). Although virtually no structural information exists, one form of PC-PLC has been purified to homogeneity from bovine seminal plasma (Sheikhnejad and Srivastava, 1986) and two others partially purified from canine myocardium (Wolf and Gross, 1985) and U937 cells (Clark et al., 1986), respectively. All these enzymes have optimal activity at neutral pH and preferentially hydrolyse phosphatidylcholine (PC). A Ca^{2+}-dependent PC-PLC activity, with neutral pH optima, has also been identified in both human and rat platelets (Nazih et al., 1990; Randell et al., 1992). Although the choline phosphate produced by PC-PLC activity has no identified signalling role (as

yet), the DAG may have functions that include the activation of PKC, and perhaps other enzymes and regulatory proteins. The hydrolysis of PC by PLC would yield DAG species that have been identified as effective PKC activators *in vitro* (Go et al., 1987; Exton, 1990), but would not yield a messenger to mobilize intracellular Ca^{2+}; the possible role of such selectivity on cellular responses is unknown.

1.4. Phospholipase A_2

It has long been suggested that the products of PLA_2 activity, a fatty acid (usually AA) released from the phospholipid *sn*-2 position and a lysophospholipid such as lyso-PC (LPC), might be involved in membrane fusion (Ahkong et al., 1973; Lucy, 1978). Both metabolites of this enzyme have been shown to increase upon stimulation of various secretory cell types including neurons (Dumuis et al., 1988; Tapia-Arancibra et al., 1992), pancreatic islets (Wolf et al., 1991; Konrad et al., 1992), mast cells (Churcher et al., 1990a), chromaffin cells (Morgan and Burgoyne, 1990) and platelets (Murayama et al., 1990). In some of these cases, PLA_2 activity has been suggested as an absolute requirement for exocytosis (Konrad et al., 1992), whereas in others it appears to play a supporting role (Metz, 1986; Rubin et al., 1991) or no role at all (Churcher et al., 1990a; Morgan and Burgoyne, 1990). Any function ascribed to PLA_2 activity must involve a role of specific lysophospholipids and/or AA, the latter of which can also

be a source of active metabolites including thromboxanes, leukotrienes, and prostaglandins (reviewed by Morgan, 1989; Nozawa et al., 1991).

1.4.1. Characterization of Cytosolic PLA_2 Species Involved in Signal Transduction

Until quite recently, only the primary sequences of two mammalian isoforms of secretory PLA_2 ($sPLA_2$) were known. Members of this widely distributed and extensively studied class of ~14 kDa PLA_2s, which are found in digestive organs and snake venoms, show no specificity for phospholipids with AA in the *sn*-2 position, and require millimolar levels of Ca^{2+} for activity (Hazen et al., 1991). Agonist-induced activation of cytosolic PLA_2 ($cPLA_2$), which catalyses AA release and thus initiates eicosanoid synthesis, has been widely characterized in various cell types (reviewed by Nozawa et al., 1991) but until recently, detailed analyses have been lacking, apparently due to low specific activities and difficulties in purification (Waite, 1987). Several groups have now successfully isolated and begun to characterize $cPLA_2$ from a variety of cells including rat kidney (Gronich et al., 1990), bovine brain (Hirashima et al., 1992) and human (Takayama et al., 1991), rabbit (Kim et al., 1991a), sheep (Hazen et al., 1991; Zupan et al., 1991) and bovine (Kim et al., 1991b) platelets. In general, these 85 – 110 kDa $cPLA_2$ share several common characteristics including neutral pH optima, a

dependence on Ca^{2+} in the physiological concentration range, and a preference for PC, phosphatidylethanolamine (PE), and in some cases, phosphatidylserine (PS), containing *sn*-2 arachidonoyl residues. Evidence for a cytosolic regulatory protein was also found (Zupan et al., 1991).

Clark et al. (1991) have now cloned and expressed a $cPLA_2$ (85.2 kDa) from U937 cells which shares no homology with known forms of $sPLA_2$. Importantly, this study demonstrates that this $cPLA_2$ translocates to membrane vesicles in response to physiologically relevant changes in $[Ca^{2+}_i]$; the domain responsible contains a 45 amino acid region with homology to part of the PKC regulatory domain (see section 1.7.). Similar putative Ca^{2+}-binding regions are found in other proteins that undergo membrane translocation and are suggested to provide a mechanism to activate $cPLA_2$ at membrane (substrate) sites relevant to second messenger functions. However, it was recently found that $cPLA_2$ activity from sheep platelets (Zupan et al., 1991), rat parotid gland (Mizuno et al., 1991) and bovine adrenal medulla (Petit et al., 1992), as well as two $cPLA_2$ isolated from bovine brain (Hirashima et al., 1992), do not require Ca^{2+} for activity.

1.4.2. Functions of PLA_2 Metabolites in Secretion

In light of the evidence, particularly from platelets, for a direct G protein-dependent regulation of PLA_2 (Fuse and Tai,

1987; Nakashima et al., 1987; Cockcroft and Stutchfield, 1989; Kajiyama et al., 1989; Silk et al., 1989; Murayama et al., 1990), it is reasonable to propose that PLA_2 activity might have a direct role in stimulus-response coupling for certain agonists such as collagen (Piche and Mahadevappa, 1989). In theory, the coupling of specific receptors to PLA_2 could permit selective cellular responses to certain agonists. These responses would be mediated by lysophospholipids (primarily LPC, LPE, LPS) and/or AA. Lysophospholipids could be functionally involved in the secretory response in several ways. First, these compounds are known to destabilize membranes *in vitro* (Howell and Lucy, 1969; Poole et al., 1970) and might therefore act in part as local fusogens in plasma and/or granule membranes. Second, lysophospholipids may act as specific enzymatic cofactors, and such a function has been demonstrated for LPC and LPA in the activation of PKC (Oishi et al., 1988; Marquardt and Walker, 1991; Asaoka et al., 1992; Van Der Bend et al., 1992b). Third, Moolenaar and colleagues have shown that LPAs having a long fatty acyl chain possess potent mitogenic activities (Van Corven et al., 1992), and have identified a putative membrane receptor that might explain the effects of LPA (Van der Bend et al., 1992a). Interestingly, LPA has been regarded as a potent platelet agonist that accumulates rapidly in intact thrombin-stimulated platelets (Gerrard and Robinson, 1989). Fourth, both LPA and LPC induce increases in $[Ca^{2+}_i]$, from internal and external sources, respectively (Jalink et al., 1990; Marquardt and Walker, 1991). Finally,

lysophospholipids could serve as sources for additional signalling molecules; lysophospholipase D activity has been identified in a number of tissues (Wykle and Strum, 1991).

The possible involvement of AA in the secretory process is substantially more complicated than that of the lysophospholipids, primarily because this lipid is largely utilized in the production of various eicosanoids that serve as intracellular second messengers or local hormones (reviewed by Morgan, 1989; Halushka et al., 1989; Nozawa et al., 1991). In particular, PGH_2 and TXA_2 are potent platelet agonist and at least one eicosanoid, lipoxin A, has been shown to activate PKC *in vitro* (see section 1.6.1.). The work of Karli et al. (1990) has also implicated an unknown AA metabolite as a potential fusogen in a cell-free fusion assay. Furthermore, similarly to the lysophospholipids, fatty acids including AA are also known to destabilize membranes (Ahkong et al., 1973; Howell et al., 1973) and might therefore serve as site-specific fusogenic lipids. As such, in combination with annexins, Ca^{2+}-dependent membrane binding proteins that promote the aggregation and contact of opposing bilayer membranes, unsaturated fatty acids promote fusion at physiologically relevant concentrations (Creutz et al., 1987). The recent identification of an annexin-like protein in human platelets (Zhuang and Stracher, 1989) makes this an intriguing model. The ability of fatty acids to directly activate PKC (see section 1.6.1.), including those species found in human platelets (Sekiguchi et

al., 187; Touny et al., 1990; Khan et al., 1991, 1992; Yoshida et al., 1992), should also be noted because of the influence of this enzyme on intracellular signalling and secretion (see section 1.6.2.). There is also substantial evidence that free fatty acids can regulate ion channels directly (reviewed by Ordway et al., 1991; also see Huang et al., 1992), in addition to the effects mediated by eicosanoids and/or PKC. This may at least in part explain the findings that polyunsaturated free fatty acids can increase $[Ca^{2+}_i]$ by mobilizing intracellular Ca^{2+} stores, independently of both PI-PLC activation and Ca^{2+} influx (Wolf et al., 1986; Chow and Jondal, 1990). Interestingly, there is evidence for substantial association of PLA_2 (unknown species), cyclooxygenase and thromboxane synthase with the dense tubular membranes of human platelets (Carey et al., 1982; Lagarde et al., 1981); this would tend to support suggested roles for AA and some metabolites in site-specific functions. Furthermore, there is some evidence that fatty acids might also have a regulatory influence on the activity of PA-phosphohydrolases (Day and Yeaman, 1992; Gomez-Munoz, 1992) and on PLD activity as well (Hattori and Kanfer, 1984; Chalifa et al., 1990). Fatty acids may also exert a feedback inhibition on PLA_2 activity (Ballou and Cheung, 1985) and the evidence indicates that this is likely to involve a direct interaction with the enzyme (Raghupathi and Franson, 1992).

In addition to these specific effects of lysophospholipids and fatty acids, as well as the numerous intra- and intercellular

effects mediated by the various eicosanoids, the more general effect of phospholipase activities (A_2, C and D) on the membrane environment, or particular microenvironments, should also be considered. It is now generally recognized that localized changes in membrane fluidity and/or lipid species distribution can influence the activities of membrane proteins, particularly receptors, enzymes and ion channels (reviewed by Curatola and Bertoli, 1987; Lenaz, 1987; Shinitzky, 1987; Henis, 1989). In light of the rapidly accumulating electrophysiological evidence that formation of a fusion pore at the site of contact between a secretory granule and the plasma membrane initiates the final step in exocytotic secretion (Almers and Breckenridge, 1988; Zimmerberg, 1988; Lindau, 1991), the presence of a proteinaceous scaffolding that requires specific lipid domains to establish a stable fusion pore across the two membranes has been suggested (Almers, 1990; Spruce et al., 1990). Yet to be determined is how this might correlate with local phospholipase-induced changes in membrane structure/composition.

1.5. Phospholipase D

Hydrolysis of PC to produce PA and free choline is catalysed by phospholipase D (PLD), an activity only recently recognized in mammalian cells (Taki and Kanfer, 1979). Over the last few years, there has been accumulating evidence for the involvement of PLD in signal transduction in a variety of

mammalian cells but purification of this enzyme activity has not yet met with success (reviewed by Exton, 1990). A mammalian plasma PLD specific for the glycosyl-phosphatidylinositol anchor moiety found on many eukaryotic cell surface proteins has, however, been purified and shown to be a single polypeptide of 110 kDa (Davitz et al., 1989). The relationship of this PLD to that/those involved in signal transduction is unknown.

1.5.1. Evidence for the Involvement of PLD In Stimulus-Response Coupling

In some cells (Billah et al., 1989; Billah and Anthes, 1990; Kennerly, 1990), the PLD pathway appears to provide a late phase of DAG production, via phosphatidate phosphohydrolase activity, whereas in other cells, including pancreatic islets (Metz and Dunlop, 1990a,b), neurons (Chalifa et al., 1990; Purkiss et al., 1991) and platelets (Rubin, 1988; van der Meulen and Haslam, 1990), it is possible that the production of PA itself is more important. In most analyses to date, PC has been identified as the principal substrate of PLD (reviewed by Billah and Anthes, 1990; Exton, 1990). However, in terms of identification of PLD activity, it is important to remember that the products of this enzyme and those of PC-PLC are interconvertible. It is fortunate, therefore, that the unique ability of PLD to catalyse a transphosphatidylation reaction between PC and a primary alcohol, with the formation of a phosphatidylalcohol rather than PA, can be

utilized as a simple, direct means of determining PLD activity (Kobayashi and Kanfer, 1987). In addition, the formation of [^{32}P]PA in cells labelled with [^{3}H]lyso-PAF, which contain [^{32}P]PC but no [^{32}P]ATP, can only occur via PLD (reviewed by Billah and Anthes, 1990; Exton, 1990). Both these methods, which have often been used in conjunction, have provided unequivocal proof of agonist-stimulated PLD activity in a number of cells.

There is now substantial evidence from a variety of cell types that nonhydrolysable GTP analogues such as guanosine 5'-O-(3-thiotriphosphate) (GTP[S]) can activate PLD. In most cells studied, an increase in [Ca^{2+}_i] also appeared to be required for receptor-linked PLD activation. However, rabbit platelet membrane preparations have been shown to contain a GTP[S]-stimulated PLD activity that is Ca^{2+}-independent, although activity was enhanced by Ca^{2+} (Van der Meulen and Haslam, 1990). A Ca^{2+}-independent PLD activity has also been identified in rat brain preparations (Taki and Kanfer, 1979) and in a neural cell line, although the latter was inhibited at higher ($\geq$ 1 μM) Ca^{2+} concentrations (Liscovitch and Eli, 1991). Although activators of PKC have been found to stimulate PLD in a range of cells, the effects of phorbol esters are attenuated by PKC inhibitors in only some instances; this has been interpreted as evidence for a possible direct effect of phorbol esters on PLD (Billah et al., 1989; Cao et al., 1990). In the light of more recent evidence, it appears that PKC can activate PLD by a mechanism independent of phosphorylation

(Conricode et al., 1992). Protein tyrosine kinase activities have also been linked to PLD activity in human neutrophils (Uings et al., 1992).

1.5.2. Possible Roles of PA in Exocytotic Secretion

PA has been reported to support and promote the aggregation and fusion of lipid vesicles *in vitro* (Nir et al., 1983; Duzgunes, 1985; Leventis et al., 1986; Papahadjopoulos et al., 1988; Park et al., 1992). In comparison to other negatively charged phospholipids, the fusion of PA vesicles has the highest sensitivity to Ca^{2+} (30–100 μM), and, quite uniquely, can be supported fully by Mg^{2+} (70–150 μM) at neutral pH; in mixtures with neutral lipids, Mg^{2+}–induced hemifusion of such vesicles is still apparent even at low (~20%) PA content. The presence of physiologically relevent concentrations of cholesterol further reduces the threshold for divalent cation–induced fusion, and can itself destabilize PA/neutral phospholipid bilayers even in the absence of divalent cations (Papahadjopoulos et al., 1988; Tilcock et al., 1988). Although these model systems may not mimic the actual events in particular membrane microenvironments, they are useful as indicators of localized membrane properties, or tendencies to destabilization (see section 1.3.2.). *In vivo*, such phenomena might be related to observations that the Ca^{2+}–dependent membrane binding of annexins is supported best by PA (Blackwood and Ernst, 1990) and that the annexin–modulated, fatty acid–

induced fusion of granule membranes is substantially potentiated by Mg^{2+} (Drust and Creutz, 1988). As the substrate of a specific PLA_2, PA could itself serve as a source of free fatty acids (Billah et al., 1981).

Murayama and Ui (1987) have postulated an interaction of PA with specific surface receptors linked to the activation of PI-PLC and PLA_2, which could explain the apparent PA-induced activation of platelets characterized by Kroll et al. (1989). However, neither of these studies effectively excluded extracellular AA or LPA as possible mediators of these results. Based on the documented growth factor-like effects of PA and LPA (Van Corven et al., 1992) and the activation of PI-PLC (Hashizume et al., 1992), this enzyme could be a genuine intracellular target of PA. In addition, PA has been found to exert both stimulatory and inhibitory influences on small GTP-binding protein regulatory factors (see section 1.2.3.) which could also result in modified phospholipase activities. Finally, there is extensive evidence that PA may modulate kinase activites. Both *in vitro* (Bell and Burns, 1991; Lee and Bell, 1992) and *in vivo* (Ando et al., 1989) studies have demonstrated that PA is an activator of PKC. That this activation occurs even in the absence of Ca^{2+} (Epand and Stafford, 1990) suggests that regulation by PA may be specific to certain species of PKC. In addition, Bocckino et al. (1991) have recently presented evidence that PA can activate other, unidentified kinase activities.

1.5.3. DAG as a Source of PA

PA can also be produced from DAG by the action of DAG-kinase (DGK); at least three species of this kinase have been identified and are known to be present in human platelets (Nozawa et al., 1991). Formation of PA by this route can easily be followed in the presence of [^{32}P]ATP; over short incubation periods the formation of [^{32}P]PA is an indicator of DAG-kinase activity. Furthermore, a specific DAG-kinase inhibitor, R-59949, has also been examined in detail. This work indicated that DGK-III and DGK-II were inhibited by this compound, but that DGK-I was insensitive (Nozawa et al., 1991). Studies with this compound have supported the role of DAG as an important second messenger in agonist-stimulated platelets (de Chaffoy de Courcelles et al., 1989), but indicate that DAG may have roles other than the activation of PKC (Rodriguez-Linares et al., 1991).

1.6. Protein Kinase C

Nishizuka and colleagues originally identified PKC as a Ca^{2+}/phospholipid-dependent protein kinase (Inoue et al., 1977), and this enzyme has proven to be an important mediator in signal transduction, regulating a variety of cellular processes often through both positive and negative feedback mechanisms (Nishizuka, 1984, 1986; Kikkawa and Nishizuka, 1986). PKC is widely distributed across vertebrate and invertebrate species and

seemingly is present in all tissues; this enzyme phosphorylates specific serine and threonine residues in a diverse range of proteins (Nishizuka, 1984). Although some of these phosphoproteins have been identified, their roles in the biological actions of PKC are in many cases still unclear.

1.6.1. Isoforms and Regulatory Characteristics

Originally described as a single polypeptide of about 80 kDa, PKC generally consists of a hydrophobic regulatory domain (~27 kDa) with binding sites for Ca^{2+}, phospholipid and DAG, and a hydrophilic, C-terminal catalytic domain (~50 kDa) that binds substrate and ATP (Parker et al., 1986). By means of hydroxyapatite chromatography and, more recently, the application of molecular biological techniques, PKC is now recognized as a family of isozymes that share, to varying degrees, common structural features, enzymatic properties/specificities, or activation requirements (Kikkawa et al., 1989; Stabel and Parker, 1991). In platelets, as in most cells, PKC is an inactive cytosolic protein that undergoes a DAG-dependent membrane translocation which reduces the $[Ca^{2+}_i]$ required for activation (reviewed by Kikkawa et al., 1989; Siess, 1989). This activation appears to involve a conformational change allowing substrate access to the catalytic site which is normally blocked by a pseudosubstrate region found in the regulatory domain (House and Kemp, 1987; Stabel and Parker, 1991). These characteristics are

common to the initially identified α, β_1, β_2, and γ isoforms, designated group A (reviewed by Stabel and Parker, 1991). Screening of cDNA libraries led to the identification of the δ, ϵ, ζ, and η isozymes (group B), which differ from the group A enzymes in regulatory domain structure and activation characteristics, in substrate specificity and in tissue/subcellular distribution (reviewed by Kikkawa et al., 1989; Stabel and Parker, 1991; Wetsel et al., 1992). In general, the type A isozymes are Ca^{2+}-dependent whereas the B-type are not. Human platelets contain PKC α and β, as well as the more recently identified δ and ζ isozymes (Crabos et al., 1991; Grabarek et al., 1992; Baldassare et al., 1992); in response to thrombin, all except δ translocate to the membrane suggesting a possible unique signalling role for this PKC isozyme.

At least three different mechanisms have been identified *in vitro* that could potentially account for PKC activation *in vivo*. As previously noted, DAG produced during the turnover of phosphoinositides increases the affinity of PKC for Ca^{2+} and phospholipid, resulting in maximal enzyme activity at physiological $[Ca^{2+}_i]$ (Kishimoto et al., 1980). Together, Ca^{2+} and DAG augment PKC activity synergistically and *sn*-1,2-DAGs, in combination with PS, have been identified as the most effective lipid activators (Nishizuka, 1984). Although this second messenger role for DAG is transient in nature, the effect of PKC activation may be prolonged, as the phosphate incorporated into PKC substrates may be relatively resistant to phosphoprotein

phosphatases (Kikkawa and Nishizuka, 1986). DAGs containing short, saturated acyl chains, as well as *sn*-1-oleoyl-2-acetyl-glycerol (OAG), are cell permeable and have been used to study the effects of PKC activation *in vivo* (Lapetina et al., 1985). In addition, PIP_2 has been identified as an activator of PKC *in vitro*, able to compete with DAG in the presence of PS and Ca^{2+} (Chauhan et al., 1989; Lee and Bell, 1991).

Available evidence strongly suggests that PKC is the principal intracellular receptor of the tumor-promoting phorbol esters (Castagna et al., 1982). Resistance to degradation has made these very potent compounds invaluable DAG mimetics for the study of PKC signalling pathways in a variety of cellular processes. Activation of PKC in intact cells by incubation with phorbol ester, synthetic DAG or Ca^{2+}-mobilizing agonists is generally associated with translocation to the particulate (predominantly membrane) phase, bringing PKC into contact with substrates. This membrane binding is Ca^{2+}-dependent, requiring increases in $[Ca^{2+}_i]$ above 100 - 200 nM (Wolf et al., 1985). Although removal of Ca^{2+} releases PKC into the cytosol, phorbol esters decrease the Ca^{2+} requirement for membrane binding and block the release of PKC upon removal of Ca^{2+}; recent studies indicate that most PKC isozymes can be activated to some degree by phorbol esters even in the absence of Ca^{2+} (Bazzi and Nelsestuen, 1989; Ryves et al., 1991).

PKC can also be irreversibly activated by soluble Ca^{2+}-dependent neutral thiol proteases called calpains in a reaction that is enhanced by PS and DAG (Kishimoto et al., 1983). Such a reaction *in vivo* would likely involve type-I calpain, which requires micromolar levels of Ca^{2+} for activation (Kuboki et al., 1992). This protease stimulates the active membrane-bound form of PKC, converting it by limited proteolysis to a Ca^{2+} and PS-independent, irreversibly activated 50 kDa enzyme (Inoue et al., 1977). Although the physiological significance of this activation remains uncertain, calpain I is known to be abundant in human platelets and, upon membrane translocation, to be activated at $[Ca^{2+}_i]$ in the physiological range (Ishii et al., 1992; Kuboki et al., 1992). As intriguing as this possible mode of PKC activation may be, the work of other groups suggests that calpain is not involved in the initial activation of platelets (Elce et al., 1989; Ariyoshi et al., 1991).

A third possible mechanism for PKC activation involves stimulation of the enzyme by unsaturated free fatty acids (reviewed by Dreher and Hanley, 1988). AA produced by PLA_2 activation, or by the action of diacyl and monoacylglycerol lipases (Prescott and Majerus, 1987), was initially proposed as a second messenger for PKC activation in the brain (Murakami and Routtenberg, 1985), but oleic acid and the AA metabolite, lipoxin A, were also shown to activate PKC *in vitro* (reviewed by Morgan, 1989). More recent studies have shown that, in the presence of

DAG, moderate concentrations of AA (10 - 30 μM) could activate the α, β, and γ isoforms of PKC (Seifert et al., 1988; Touny et al., 1990; Khan et al., 1991; Kitagawa et al., 1991; Shearman, et al., 1991; Shinomura et al., 1991). Similar results have been obtained in preparations of *Hermissenda* photoreceptors (Lester et al., 1991) and neuroblastoma cells (Larsson et al., 1992). In addition, it has recently been reported that PC, which has an inhibitory effect on PS/DAG-induced PKC activity (Kaibuchi et al., 1981), can support the activation of α, β, and γ isozymes by the synergistic combination of DAG and oleic acid (Chen et al., 1992). Lee and Bell (1992) have also obtained evidence that implicates PA as a potentially important regulator of PKC activity. These studies suggest that (i) activation of PLC, PLA_2 and/or PLD might induce a synergistic activation of (specific) PKC isozymes; and (ii) PC and PA may have roles in the differential regulation of PKC isozymes.

In addition to the above mechanisms, the activity of PKC may also be subject to negative regulation. Autophosphorylation may inhibit enzyme activity (Flint et al., 1990), and has also been implicated in the release of activated PKC from the membrane (Wolf et al., 1985). It is also possible that the different PKC isozymes may regulate the activity of one another by phosphorylation (Dreher and Hanley, 1988). In addition, sphingosine and gangliosides may also regulate PKC, since these compounds have been shown to attenuate enzyme activity *in vitro*

(Hannun et al., 1986).

1.6.2. Role of PKC in Stimulus - Secretion Coupling

Studies employing phorbol esters and synthetic DAG have proven that PKC has an important role in the secretory response of a variety of cells including platelets. Such compounds have been shown to cause aggregation and slow secretion, but not shape change, in human platelets (Kaibuchi et al., 1983; Lapetina et al., 1985). At concentrations that have little effect alone, the combination of Ca^{2+} ionophore and phorbol ester produces maximal platelet aggregation and secretion, similar to that observed with thrombin (Kaibuchi et al., 1983; Yamanishi et al., 1983). Platelet activation by thrombin and collagen is accompanied by substantial phosphorylation of both a 40 - 47 kDa protein (P47) and the 20 kDa myosin light-chain (MLC) (Lyons et al., 1975; Haslam and Lynham, 1977). Although it has been purified (Imaoka et al., 1983) and cloned (Tyers et al., 1988), the identity and function of P47 has not yet been determined. Based on its specific expression in hematological cells the protein has been named pleckstrin for platelet and leukocyte C kinase substrate, and for KFARKSTRRSIR, the most probable phosphorylation site (Tyers et al., 1988). Studies have demonstrated that pleckstrin is the major platelet substrate of PKC (Nishizuka, 1984). During platelet activation, MLC is phosphorylated by Ca^{2+}/calmodulin-

dependent MLC-kinase (Daniel et al., 1984) leading to granule centralization and exocytosis (Painter and Ginsberg, 1984). PKC also phosphorylates MLC, but at a different site from MLC-kinase (Naka et al., 1983). The significance of these different phosphorylation sites is unknown but suggest alternate regulatory pathways in the control of MLC during secretion. It has been proposed that Ca^{2+} mobilization and PKC activation interact synergistically to produce an optimal platelet response (Nishizuka, 1984); this model might account fully for dense granule secretion (Haslam et al., 1985; Yamanishi et al., 1983) but only partially for lysosomal enzyme release (Kajikawa et al., 1983; Knight et al., 1984).

In addition to promoting stimulus-response coupling, PKC can also regulate signalling events by feedback mechanisms that primarily inhibit the production of second messengers. In a wide variety of cells, including platelets, phorbol ester treatment has been shown to block PLC activity (Abdel-Latif, 1986; Zavoico et al., 1985; Crouch and Lapetina, 1988; Bishop et al., 1990), and this effect was reversed by PKC inhibitors. The actions of phorbol esters on agonist-stimulated PI-PLC activity are selective and there have been suggestions that receptor phosphorylation by PKC may mediate these effects; phosphorylation of PLC by PKC has been demonstrated (Bennett and Cooke, 1987), leading to speculation that phosphorylation regulates the membrane translocation and thus activation of PLC. In addition, the G

proteins coupling receptors to PLC could also be PKC substrates. The PKC-induced phosphorylation of G_z in response to thrombin, U46619 and phorbol ester has been documented, but the significance in terms of G protein function remains unknown (Manning and Brass, 1991).

In addition to PKC activity, thrombin and other agonists stimulate substantial tyrosine phosphorylation in a variety of unidentified platelet proteins (Golden and Brugge, 1989; Nakamura and Yamamura, 1989). Tyrosine phosphorylation/dephosphorylation has recently come under intense study, but there is as yet no definitive evidence for a key role of these activities in the initiation of platelet activation and secretion. Five src-related protein tyrosine kinases (PTKs) have been identified in human platelets: src, fyn, yes, hck and two variants of lyn proteins (Ferrell et al., 1990; Huang et al., 1991a). Fyn, lyn and yes proteins were found to be physically associated with the membrane glycoprotein GPIV (CD36), which may be a binding site for thrombospondin and/or collagen (Huang et al., 1991a); these findings suggest a possible intracellular signalling role for PTKs during aggregation. However, the work of Yamamoto et al. (1990) suggests that any such function is not essential to normal platelet function. Interestingly, fyn, lyn and yes proteins have also been found associated with a ras GAP protein in thrombin-stimulated platelets (Cichowski et al., 1992), but the functional significance of this in platelet signalling is unknown. Bachelot

et al. (1992) have concluded that PTKs are activated subsequent to PKC, and that protein tyrosine phosphorylation is associated with strong aggregation, occurring late during stimulation with thrombin or collagen, and/or with the activation of GPIIb-IIIa. These results suggest that the activity of PTKs may be modulatory in nature, and not essential to platelet activation. Furthermore, use of the inhibitors vanadate and molybdate in electropermeabilized platelets has implicated tyrosine phosphatases and a 50 kDa phosphoprotein in the Ca^{2+}-dependent secretion of both dense and α-granule constituents (Lerea et al., 1989). However, it would appear that these secretory responses were mediated by the activation of PI-PLC and therefore could not be definitively linked to tyrosine phosphorylaton. Additionally, these inhibitors could not fully mimic the effects of a maximal dose of GTP[S] on secretion from dense granules. Therefore, it is somewhat unlikely that tyrosine phosphorylation/dephosphorylation mediates the effects of a putative G_E in platelets.

1.7. Phosphoprotein Phosphatases

The reversible phosphorylation of proteins has been recognized as a major mechanism of second messenger action in eukaryotic cells; phosphorylation/dephosphorylation at serine, threonine and tyrosine residues elicits conformational and thus functional changes in regulated proteins (reviewed by Cohen, 1989; Tonks and Charbonneau, 1989). Whereas substantial attention has

been given to protein phosphorylation, dephosphorylation mechanisms inherent to intracellular signalling have, until relatively recently, been less widely studied.

1.7.1. Species and Regulatory Characteristics

Four major serine/threonine-specific protein phosphatase catalytic subunits are present in eukaryotic cells (reviewed by Morgan, 1989; Cohen, 1991). These phosphatases are grouped into four major classes termed PP-1, 2A, 2B, and 2C according to (i) their ability to dephosphorylate specifically the β (type 1) or α (type 2) subunits of phosphorylase kinase; and (ii) their sensitivity (type 1) or insensitivity (type 2) to nanomolar concentrations of two heat and acid-stable proteinaceous inhibitors (I-1 and I-2). Subdivision of type 2 phosphatases was based primarily on divergent dependencies on divalent cations; PP-2B and 2C have absolute requirements for Ca^{2+} and Mg^{2+}, respectively, whereas PP-2A and PP-1 are generally active in the absence of divalent cations. PP-2A are also recognized for their stimulation by polycationic compounds. These criteria and others have been reviewed by Cohen (1989). PP-1, 2A, and 2B are members of the same gene family, whereas PP-2C appears to be distinct. These four classes of protein phosphatase also differ in their specificity toward small phosphorylated peptides, with PP-1 and 2B displaying negligible activities, suggesting that they recognize higher order structural features. PP-2A and 2C do however readily

dephosphorylate a variety of small phosphopeptides (Agostinis et al., 1987).

PP-1, 2A, and 2C have broad, overlapping specificities *in vitro*, and account for almost all measureable phosphatase activity related to intracellular functions (reviewed by Cohen, 1989). The subcellular locations of these phosphatases, their regulatory mechanisms, and their precise functions *in vivo* are, however, largely unknown. PP-1 exists in an inactive cytosolic form and an active form that is largely associated with the particulate fraction of cell lysates. This phosphatase is subject to inhibition by cAMP through phosphorylation of I-1 by cAMP-dependent protein kinase; dephosphorylation of I-1 by PP-2B, in the presence of increased $[Ca^{2+}_i]$, can activate PP-1. PP-2B (calcineurin) is Ca^{2+}/calmodulin-dependent and has been found in both particulate and cytosolic platelet fractions; limited proteolysis produces calmodulin-independent activity (Tallant and Wallace, 1985). Although the potential for activation of PP-2A by polycationic compounds has proven to be quite substantial, the actual *in vivo* regulatory mechanisms of this enzyme and PP-2C remain unknown.

1.7.2. Role of Phosphatases in Exocytosis

Although the precise functions of most protein phosphatases remains vague, important clues to physiological roles have been

provided by general inhibitors such as 1-naphthylphosphate which, in addition to effects on acid and alkaline phosphatases, has also been shown to inhibit PP-1 and 2A (Pondaven and Meijer, 1986). Using this compound, Wagner and Vu (1990) demonstrated a substantial enhancement of Ca^{2+}-independent secretion from PC12 cells. In addition, the cell-permeable inhibitors okadaic acid (OKA) and calyculin A (CAL), specific for PP-1 and 2A, have proven useful in examining the role(s) of protein phosphatases in exocytosis. With OKA, Karaki et al. (1989) found an inhibition of thrombin-induced platelet activation. Murata et al. (1992) claimed that the enhanced phosphorylation of a 50 kDa protein was related to inhibition of platelet activation. Although no phosphorylation of this protein was evident under control conditions, these authors have suggested that PP-1 might have a priming effect on platelet activation. Similarly, modulatory roles for PP-1 and/or PP-2A in both neutrophil activation (Lu et al., 1992) and neurotransmitter release (Abdul-Ghani et al., 1991) have also been suggested. In addition, Jena et al. (1991) have shown that protein tyrosine dephosphorylation may be involved in the regulation of Ca^{2+}-dependent secretion from pancreatic acini, and that much of this phosphatase activity is associated with zymogen granule membranes. In this respect, it is interesting that a pertussis toxin-sensitive G protein has now been shown to regulate a membrane-associated phosphotyrosine phosphatase activity in a pancreatic cell line (Pan et al., 1992). Notably, the immunosuppressive agents FK506 and cyclosporin A block

exocytosis in a rat basophilic leukemia cell line (Hultsch et al., 1991) and it has recently been shown that these agents, in combination with specific immunophilins (immunosuppressant-binding proteins), inhibit the Ca^{2+}/calmodulin-dependent phosphatase calcineurin (Liu et al., 1991). A calcineurin-like phosphatase has also been characterized in human platelets (Tallant and Wallace, 1985).

1.8. The Electropermeabilized Platelet

The reversible electrical breakdown of biological membranes was discovered by accident ~20 years ago and its practical application in the study of intracellular events was immediately recognized by several groups (reviewed by Knight and Scrutton, 1986; Zimmerman and Stopper, 1987). Although special equipment is required, this approach is biochemically clean and produces small (~2 nm diameter) stable pores in the plasma membrane (provided the permeabilized platelets are maintained at ~4°C). Thus, only low-M_r solutes can exchange between the cytosol and the extracellular medium. Since changes in platelet volume occur after electropermeabilization, and such swelling must necessarily involve cytoskeletal disruption and externalization of plasma membrane invaginations, it has been suggested that these effects might in part account for the slower secretion characteristic of this preparation (Haslam and Davidson, 1984c). However, there is no doubt that, upon warming and stimulation, these preparations

release their granule contents via mechanisms involving genuinely selective exocytotic secretion (Knight and Scrutton, 1980, 1986; Haslam and Davidson, 1984a). In addition, the introduction of gel filtration to remove free low-M_r cytosolic factors, subsequent to electropermeabilization, further improved the methodology, yielding "cleaner" homogeneous platelet suspensions for experimental use (Haslam and Davidson, 1984a). The stability of these preparations allows all experimental additions to be thoroughly equilibrated with the platelet interior. Buffering systems, generally EGTA/EDTA - based, are used to fix the concentrations of divalent cations (principally Ca^{2+} and Mg^{2+}), thus preventing alterations by internal sequestration and/or mobilization mechanisms; ATP is also included in most preparations.

1.8.1. Thrombin, Calcium and Guanine Nucleotides in the Initiation and Regulation of Secretion

As outlined earlier (see section 1.1. for example) there is now substantial evidence that Ca^{2+} is a major intracellular messenger in stimulus-secretion coupling. In a number of secretory cells, including platelets, the use of electropermeabilized preparations has been central to the characterization of Ca^{2+}-dependent secretory responses. Baker and Knight (1978) were the first to characterize Ca^{2+}-dependent secretion in electropermeabilized bovine adrenal medullary cells.

Shortly thereafter, Knight and Scrutton (1980) characterized the role of Ca^{2+} in secretion from electropermeabilized platelets. Half-maximal secretion was seen with ~2 μM Ca^{2+}_{free}, and a much higher concentration of Ca^{2+} was required to elicit maximal release. This suggested the involvement of additional factors that could increase the sensitivity of the secretory response to this cation *in vivo*. These original findings have been confirmed by Haslam and Davidson (1984a), who initiated investigations into factors that might enhance the response to Ca^{2+}. Thrombin was found to enhance greatly the Ca^{2+} sensitivity of secretion from permeabilized platelets, and this effect was most pronounced at ~1 μM [Ca^{2+}_{free}] (Haslam and Davidson, 1984a; Knight and Scrutton, 1984). This secretion correlated with the formation of DAG, the phosphorylation of the PKC substrate pleckstrin (Haslam and Davidson, 1984c) and the breakdown of polyphosphoinositides (Culty et al., 1988). These findings were consistent with the hypothesis that thrombin activation of PI-PLC, leading to Ca^{2+} mobilization and PKC activation, might explain the secretion of dense granule constituents (Haslam and Davidson, 1984b; Haslam et al., 1988). In support of this hypothesis, Haslam and colleagues (Haslam and Davidson, 1984c; Haslam et al., 1985) demonstrated that GTP potentiates the stimulatory action of thrombin and that nonhydrolysable GTP analogues themselves substantially enhance the Ca^{2+}-sensitivity of 5-HT secretion, suggesting that a GTP-binding protein might play a role in PI-PLC activation. Furthermore, the DAG mimetics OAG and phorbol ester were also shown to enhance the

Ca^{2+}-sensitivity of secretion in this system (Knight et al., 1984).

The stimulatory effects of thrombin on DAG production and 5-HT secretion from electropermeabilized human platelets have also been observed with PAF, vasopressin and a prostaglandin endoperoxide analogue (U46619) (Haslam and Davidson, 1984b,c). Since the stimulatory actions of these agonists were apparent even at 0.1 μM Ca^{2+}_{free}, these studies suggested that PI-PLC could have significant activity under conditions equivalent to the basal $[Ca^{2+}_i]$ of intact platelets. There is ample evidence from intact platelet studies that different agonists utilize the PI-PLC pathway either to different extents, or not at all (see section 1.1.). Thus, whereas thrombin and PAF activate PI-PLC, ADP acts primarily to increase Ca^{2+} entry and epinephrine appears to have little effect on either phosphoinositide breakdown or $[Ca^{2+}_i]$ (reviewed by Haslam, 1987). In accord with these findings, neither epinephrine nor ADP reduced the $[Ca^{2+}_{free}]$ required for secretion from electropermeabilized platelets (Knight and Scrutton, 1985). The secretion of lysosomal constituents appeared to be less sensitive to the activation of PI-PLC than did that from dense granules (Knight et al., 1982, 1984).

Studies on saponin-permeabilized platelets also suggested the involvement of G proteins in receptor-mediated platelet activation, and this system has provided evidence that the

activation of PLA_2 may be an alternative (or coincident) mechanism to PI-PLC - mediated platelet responses (see section 1.4.). In addition, findings in electropermeabilized platelets showed that an inhibitory GTP analogue (GDP[S]), which markedly diminished the effects of guanine nucleotides, had little effect on thrombin-induced secretion (Haslam and Davidson, 1984b,c; Haslam et al., 1988), suggesting possible G protein - independent pathways. Furthermore, the available data from electropermeabilized platelets suggested that cAMP exerted its inhibitory effects on secretion at some point prior to PLC activation (reviewed by Knight and Scrutton, 1986; Haslam et al., 1988).

1.8.2. Relationship Between Protein Phosphorylation and Secretion

Although specific patterns of protein phosphorylation have long been recognized to correlate with secretion from human platelets (reviewed by Haslam et al., 1980) the functional significance of these phosphoproteins in relation to secretion remains largely unknown. In particular, the major PKC substrate in platelets, pleckstrin, and the light chain of myosin (P20), are often phosphorylated in stimulated platelets, the latter by the Ca^{2+}/calmodulin-regulated myosin light chain kinase and PKC (reviewed by Haslam, 1987). However, studies in permeabilized platelets have shown that substantial pleckstrin phosphorylation occurs at concentrations of Ca^{2+} and thrombin too low to induce

more than a minor secretory response (Haslam and Davidson, 1984a). In addition, Rittenhouse and Horne (1984) have reported secretion from dense granules in the absence of both PI-PLC activity and pleckstrin phosphorylation. Thus, although pleckstrin phosphorylation has proven quite useful as an endogenous index of PKC activity, these studies have also called into question any primary role for PKC in secretion. In addition, alternative (fusogenic?) roles for DAG have not been ruled-out in these platelet preparations. Furthermore, original evidence that ATP may not be absolutely essential to the secretory response proper, but rather necessary for maintenance of response competence (Knight and Scrutton, 1980), suggested that protein kinase activity is not an essential component of exocytotic secretion. In light of this, 5-HT secretion from saponin-permeabilized platelets was not prevented by prior depletion of ATP (Ruggiero et al., 1985). This study and others (Rittenhouse and Horne, 1984; Lapetina et al., 1985) suggested that both thrombin and collagen could induce platelet 5-HT secretion in the absence of PI-PLC activity, PKC activity and cyclooxygenase products. Although some important controls were absent from these studies (e.g. no measurements of DAG), the findings are important in the light of what has been learned in other systems, and suggest that alternative mechanisms, in addition to the activation of PI-PLC, could also be involved in agonist-induced secretion. Indeed, a role for protein phosphorylation in the terminal stage(s) of exocytosis in many secretory cells has yet to be proven

uequivocally, although a role in priming the mechanism seems certain (Baker, 1988; Knight et al., 1988; Holz et al., 1989; Churcher and Gomperts, 1990).

1.8.3. Evidence for G_E in the Regulation of Secretion

Until the mid-to-late 1980's it was generally accepted that stimulus-secretion coupling was a Ca^{2+}-dependent process mediated largely (if not entirely) through the bifurcating signal transduction pathways arising from the receptor-mediated activation of PI-PLC. There were exceptions however, including the secretion of parathyroid hormone which responded to a decrease in $[Ca^{2+}_i]$ (Muff and Fischer, 1986). With the adoption of a variety of permeabilization procedures (see section 1.1.), it became possible to directly control the cytosolic composition. Initially, this approach appeared to largely confirm the Ca^{2+}-dependence of secretion from a variety of cells, including platelets (see section 1.8.1.). Fernandez et al. (1984) were the first to demonstrate that GTP[S] could cause secretion from mast cells in the absence of increases in $[Ca^{2+}_i]$. Shortly afterwards, Barrowman et al. (1986) demonstrated an additive effect of low Ca^{2+} and GTP (analogue) concentrations in permeabilized neutrophils, similar to that described in permeabilized platelets (Knight and Scrutton, 1980; Haslam and Davidson, 1984a); GDP and analogues inhibited stimulation by Ca^{2+} alone, leading Gomperts and colleagues (Gomperts et al., 1986) to speculate on the

1984a); GDP and analogues inhibited stimulation by Ca^{2+} alone, leading Gomperts and colleagues (Gomperts et al., 1986) to speculate on the existence of another GTP-binding protein closer to the final exocytotic event. With the demonstration that GTP[S] could induce secretion from permeabilized adrenal chromaffin cells (Bittner et al., 1986), mast cells (Cockcroft et al., 1987) and RINm5F cells (Vallar et al., 1987) in the absence of PI-PLC activity, the concept of a putative G_E mediating exocytosis became even more widely accepted. Ca^{2+}-independent secretion stimulated by GTP[S] has also more recently been demonstrated in chromaffin cells (Morgan and Burgoyne, 1991) and an anterior pituitary cell line (AtT-20; Guild, 1991). Furthermore, Howell et al. (1987) showed that ATP was not necessary for exocytosis from permeabilized mast cells stimulated by Ca^{2+} and GTP[S], and similar findings have been obtained in HL60 cells (Stutchfield and Cockcroft, 1988) and neutrophils (Cockcroft, 1991). These results suggested a modulatory rather than essential role for protein phosphorylation in the regulation of secretion, and indeed, Churcher et al. (1990) have presented evidence to suggest that a dephosphorylation reaction may be mediated by G_E in the terminal stages of exocytosis. Although all these studies strongly suggested that G_E did exist and had a fundamental role in secretion from a variety of cell types, the evidence from platelets, was generally inconclusive and provided little evidence for a role of G_E in secretion from dense granules (see section 1.9.1.).

1.9. Rationale for the Research Undertaken (late 1988)

The term fusion, as it applies to membranes *in vivo*, implies the occurrence of topological changes requiring two critical steps: (i) the very close approach of the two membranes (adherence); and (ii) the destabilization, rupture and coalescence of the two membranes only in the contact area (Rand and Parsegian, 1986). By definition, exocytosis involves fusion and the best evidence to date implicates the formation of a fusion pore between apposed granule and plasma membrane sites as the trigger event leading to the extrusion of granule constituents (Almers, 1990; Lindau, 1991). By necessity, extrusion must involve the coalescence of the granule and plasma membranes, which is suggested to proceed on the scaffolding provided by the fusion pore; "permissive" lipid microenvironments in each membrane are deemed by some to be essential for such a process (Monck et al., 1990; Spruce et al., 1990).

1.9.1. Factors Affecting Secretion from Dense and α-Granules

In view of the importance of α-granule constituents to hemostasis, and perhaps other physiological processes, remarkably little was known regarding the secretion of these compounds from platelets, even though α-granules are by far the most numerous of the three granule types present (reviewed by Weiss, 1982; Kaplan, 1986). In a comparison of the effects of various aggregating

agents (including thrombin, collagen, ADP and epinephrine), Kaplan et al. (1979) found that the constituents of both dense and α-granules had a similar time-course for release. This study and others (Holmsen et al., 1982; Holmsen, 1985) also provided some evidence to suggest that secretion from all three granule types (dense, α and lysosomes) might be somewhat selective and therefore perhaps differentially regulated. Evidence for the preferential secretion of dense granule constituents in comparison to lysosomal enzymes was also presented by Knight and Scrutton (1984). Furthermore, the results of Akkerman et al. (1982) suggested that dense rather than α-granules were more readily released, although this result was opposite to the findings of Kaplan et al. (1979). Since two studies characterizing the selective release of different granule types from the neutrophil had just been completed (Barrowman et al., 1986, 1987; Lew et al., 1986), it seemed possible that similar mechanisms might be at work in the platelet. Thus, the initial research objective of this thesis was a comprehensive comparative study of the factors regulating dense and α-granule secretion. Could alternative secretory pathways be involved? The electropermeabilized platelet seemed to be an ideal model with which to address this issue.

Evidence from the neutrophil studies discussed above, in addition to reports of Ca^{2+}/PI-PLC - independent secretion in several cell types and conflicting evidence for the involvement of PKC in exocytosis, suggested that G_E might mediate secretion from

particular granules in some cell types (reviewed by Gomperts, 1990). Although the available evidence suggested that there was little Ca^{2+}-independent secretion of dense granule constituents from electropermeabilized human platelets (Haslam and Davidson, 1984b; Knight and Scrutton, 1986b), these results could have been due to incomplete buffering of residual endogenous Ca^{2+} by EGTA, to the use of submaximal guanine nucleotide concentrations, or the presence of a Ca^{2+} chelator (BAPTA) with toxic effects (Knight et al., 1989). Thus, a possible role for G_E in secretion from dense granules had not been totally excluded and the involvement of such a G protein had not even been investigated in relation to the secretion of α-granule constituents. In addition, phorbol esters were known to induce slow, Ca^{2+}-independent secretion from both intact (Rink et al., 1983) and electropermeabilized platelets (Knight and Scrutton, 1984), and the presence of Ca^{2+}-independent species of PKC in human platelets had also been reported (Tsukuda et al., 1988). Based on this information, studies involving optimal concentrations of both GTP[S] and a phorbol ester (PMA), as well as GTP and thrombin, were carried out in the effective absence of Ca^{2+}, as well as over a range of buffered free Ca^{2+} concentrations. A commercially available radioimmunoassay for the α-granule constituent β-thromboglobulin was adapted for use in these experiments. This initial study (Paper 1; Coorssen et al., 1990) supported a possible role for the putative G protein, G_E, in secretion from both dense and α-granules.

1.9.2. The Role of PKC in Secretion

PKC inhibitors were employed with the aim of isolating any PKC-independent effects of GTP[S] and to examine the relationship between protein phosphorylation and secretion in more detail. The specific role of PKC in platelet secretion would be evident from such inhibitor studies, as would any other possible effects of DAG (whether produced by PI- or PC-PLC). These studies (Paper 2) confirmed that interacting effects of Ca^{2+}, PKC and a G protein-activated mechanism mediate secretion from both dense and α-granules. No single factor was essential to the exocytotic mechanism and any combination of two could produce marked secretory responses. Furthermore, the evidence indicated that GTP[S] could elicit secretion by a mechanism additional to the activation of PKC, as suggested for thrombin in earlier studies (Lapetina et al., 1985). Might a protein phosphatase be involved? Before invoking the existence of two (or more) effectors, evidence concerning other candidate enzymes that fulfilled the criteria for activation under the established experimental conditions were considered.

1.9.3. Correlations Between PLD Activity and Secretion

The findings presented in Papers 1 and 2 established that the activities of both PI- and PC-PLC could be dissociated from the exocytotic mechanism. Moreover, the evidence available at the

time indicated that PLA_2 was a Ca^{2+}-dependent enzyme, the activity of which had also been dissociated from the secretory process in mast cells (Churcher et al., 1990a). In view of (i) the synergistic (GTP[S] + PMA) and Ca^{2+}-independent activation of PLD in rabbit platelet membranes (van der Meulen and Haslam, 1990), (ii) correlations between mast cell secretion and PLD activity (Gruchalla et al., 1990), (iii) the support of PKC activation by PA (Kaibuchi et al., 1981; Epand and Stafford, 1990), (iv) the possible regulation of low-M_r GTP-binding proteins by PA (Takai et al., 1992) and (v) the possible fusogenic capacity of PA (Leventis et al., 1986; Papahadjopoulos et al., 1988), PLD was considered to be the most promising effector candidate. Labelling conditions that permitted a study of PLD activity in human platelets were established and extensive correlative evidence for a relationship between PLD activity (PA formation) and the secretion of dense granule constituents from electropermeabilized platelets was obtained (Paper 3). In all cases, PLD activity was confirmed by the formation of PEt in the presence of ethanol. BAPTA and its analogues were identified as inhibitors of PLD and their effects characterized; the effects of these compounds strengthened the correlation between PLD activity and secretion (Paper 4). PA-phosphohydrolase was found to have little activity in this system.

1.9.4. PLA_2 Activity and Secretion

With recent reports that AA could promote the activation of

PKC, particularly in the presence of other stimulatory lipids (Khan et al., 1991; Shinomura et al., 1991) and the identification of a Ca^{2+}-independent species of $cPLA_2$ in sheep platelets (Zupan et al., 1991), it became evident that a possible role for PLA_2 in the secretory mechanism required direct investigation. Exogenous AA was found to promote GTP[S]-induced secretion in the absence of Ca^{2+}, independently of the formation of lipoxygenase or cyclo-oxygenase metabolites, and a GTP[S]-stimulated Ca^{2+}-independent PLA_2 activity was found in the electropermeabilized platelet. Specific PLA_2 inhibitors (and activators) were identified and used to prove that neither AA nor its metabolites have an essential role in the exocytotic mechanism (Paper 5). Only inhibitors that blocked PLD as well as PLA_2 had any effect on secretion. Further studies will need to be undertaken to determine whether the PA produced by PLD is different from that formed by the PLC/DAG kinase pathway (either in terms of the fatty acids present and/or of site-specific production), before a distinct role for PLD in secretion can be postulated. Possible functions of PA, other than the activation of kinases, must also be considered.

PAPER I

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Factors affecting dense and α-granule secretion from electropermeabilized human platelets: Ca^{2+}-independent actions of phorbol ester and GTPγS

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Electropermeabilized human platelets containing 5-hydroxy[^{14}C]tryptamine ([^{14}C]5-HT)[1] were suspended in a glutamate medium containing ATP and incubated for 10 min with (in various combinations) Ca^{2+} buffers, phorbol 12-myristate 13-acetate (PMA), guanine nucleotides, and thrombin. Release of [^{14}C]5-HT and β-thromboglobulin (βTG) were used to measure secretion from dense and α-granules, respectively. Ca^{2+} alone induced secretion from both granule types; half-maximal effects were seen at a −log [Ca^{2+}_{free}] (pCa) of 5.5 and maximal secretion at a pCa of 4.5, when ~80% of 5-HT and ~50% of βTG were released. Addition of PMA, guanosine 5'-O-(3-thiotriphosphate) (GTPγS), GTP, or thrombin shifted the Ca^{2+} dose-response curves for secretion of both 5-HT and βTG to the left and caused small increases in the maximum secretion observed. These results suggested that secretion from α-granules, like that from dense granules, is a Ca^{2+}-dependent process stimulated by the sequential activation of a G-protein, phospholipase C, and protein kinase C (PKC). However, high concentrations of PMA and GTPγS had distinct effects in the absence of Ca^{2+} (pCa > 9); 100 nM PMA released ~20% of platelet 5-HT but little βTG, whereas 100 μM GTPγS stimulated secretion of ~25% of each. Simultaneous addition of PMA greatly enhanced these effects of GTPγS. Phosphorylation of pleckstrin in permeabilized platelets incubated with [γ-^{32}P]ATP was used as an index of the activation of PKC during secretion. In the absence of Ca^{2+}, 100 nM PMA caused maximal phosphorylation of pleckstrin and 100 μM GTPγS was ~50% as effective as PMA; neither GTPγS nor Ca^{2+} enhanced the phosphorylation of pleckstrin caused by 100 nM PMA. These results indicate that, although activation of PKC promoted secretion, GTPγS exerted additional stimulatory effects on secretion from both dense and α-granules that were not mediated by PKC. Measurement of [^{3}H]inositol phosphate formation in permeabilized platelets containing [^{3}H]phosphoinositides showed that GTPγS did not stimulate phosphoinositide-specific phospholipase C in the absence of Ca^{2+}. It follows that in permeabilized platelets, GTPγS can both stimulate PKC and enhance secretion via G-protein–linked effectors other than this phospholipase.

Introduction

Platelets possess three types of secretory organelle: dense granules containing nucleotides, 5-hydroxytryptamine (5-HT), and Ca^{2+}; lysosomes containing acid hydrolases; and α-granules, which are present in much larger numbers than the others and contain numerous proteins with diverse functions (Zucker and Nachmias, 1985). Secreted α-granule constituents include β-thromboglobulin (βTG), platelet factor 4, fibrinogen, thrombospondin, von Willebrand factor, fibronectin, factor V, platelet-derived growth factor, and protease nexin-II (Kaplan *et al.*, 1979; Kaplan, 1986; Sander *et al.*, 1983; Stenberg *et al.*, 1984; Wencel-Drake *et al.*, 1985; Van Nostrand *et al.*, 1990). Despite the importance of some of these proteins in platelet aggregation (fibrinogen and thrombospondin), adhesion of platelets to the subendothelium (von Willebrand factor, fibronectin), formation of the prothrombinase complex (factor V), various cell growth responses (platelet-derived growth factor, protease nexin-II), and chemotaxis (βTG and platelet factor 4), much less is known about the factors that regulate α-granule secretion than about dense granule secretion (Kaplan, 1986). However, release of dense granule constituents by aggregating agents such as ADP, epinephrine, arachidonate, and low concentrations of

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[1] Abbreviations: βTG, β-thromboglobulin; DAG, 1,2-diacylglycerol; EGTA, ethylene glycol-bis(β-aminoethyl ether)-*N,N,N',N'*-tetraacetic acid; GTPγS, guanosine 5'-O-(3-thiotriphosphate); 5-HT, 5-hydroxytryptamine; pCa, −log [Ca^{2+}_{free}]; PKC, protein kinase C; PMA, phorbol 12-myristate 13-acetate; RIA, radioimmunoassay.

thrombin or collagen is associated with the release of α-granule proteins, and it is clear that the high thrombin and collagen concentrations needed to release lysosomal hydrolases are not required (Kaplan *et al.*, 1979). Indeed, there is evidence that low concentrations of aggregating agents may release a higher percentage of α-granule proteins than of dense granule 5-HT (Kaplan *et al.*, 1979), though others have found that α-granule secretion is slower than that from dense granules (Akkerman *et al.*, 1982).

Studies on electropermeabilized human platelets have provided detailed information on intracellular factors that regulate the secretion of dense granule 5-HT and of lysosomal enzymes. High concentrations of Ca^{2+} ions (−log [Ca^{2+}_{free}] [pCa] 5) promote secretion of 5-HT in the presence of ATP (Knight and Scrutton, 1980). Phorbol ester (Knight and Scrutton, 1984), thrombin (Haslam and Davidson, 1984a; Knight and Scrutton, 1984), and guanine nucleotides—especially guanosine 5'-*O*-(3-thiotriphosphate) (GTPγS) (Haslam and Davidson, 1984b; Knight and Scrutton, 1986)—cause a leftward shift in the Ca^{2+} dose-response curve and thus greatly potentiate secretion at Ca^{2+} concentrations in the physiological range (pCa 7 to 6). These results can be at least partly explained by a G-protein–mediated activation of phosphoinositide-specific phospholipase C and the formation of 1,2-diacylglycerol (DAG) that promotes secretion by stimulating protein kinase C (PKC) (Haslam and Davidson, 1984b,c; Knight and Scrutton, 1984, 1986; Culty *et al.*, 1988). The release of lysosomal enzymes from electropermeabilized platelets is also stimulated by Ca^{2+}, but in this case thrombin, guanine nucleotides, and a synthetic DAG have been shown to enhance secretion primarily by increasing the maximum response to Ca^{2+} rather than the sensitivity of the secretory mechanism to this ion (Knight *et al.*, 1984; Athayde and Scrutton, 1990). Apart from preliminary reports (Coorssen and Haslam, 1990; Peltola and Scrutton, 1990), comparable information on the factors regulating α-granule secretion is not yet available. We present here a detailed comparison of dense and α-granule secretion from permeabilized human platelets, which shows that the same factors promote secretion from both granule types, though not with equal effectiveness.

Studies on other permeabilized cells have suggested that guanine nucleotides can stimulate secretion by a mechanism additional to the sequential activation of phospholipase C and PKC (reviewed by Gomperts, 1990). Thus, the effects of GTPγS on secretion from neutrophils and HL60 cells differ from those observed after direct activation of PKC by phorbol 12-myristate 13-acetate (PMA) (Barrowman *et al.*, 1986; Stutchfield and Cockcroft, 1988). Moreover, GTPγS can induce secretion without formation of phosphoinositide breakdown products in permeabilized adrenal chromaffin cells (Bittner *et al.*, 1986), mast cells (Cockcroft *et al.*, 1987), and RINm5F cells (Vallar *et al.*, 1987). These studies led Gomperts and colleagues to propose that in many cells a GTP-binding protein (termed G_E), distinct from that mediating the activation of phospholipase C, may play an essential role in the exocytotic event (Barrowman *et al.*, 1986; Cockcroft *et al.*, 1987; Howell *et al.*, 1987; Gomperts, 1986, 1990). Initial interpretations of studies with permeabilized platelets did not support a role for G_E in dense granule secretion, because GTPγS appeared to have little effect in the absence of Ca^{2+} ions (Haslam and Davidson, 1984b; Knight and Scrutton, 1986). However, in the present study, we have reexamined this question and have demonstrated that GTPγS, acting either alone or more potently in combination with PMA, can induce a Ca^{2+}- and phospholipase C-independent secretion of both dense and α-granule constituents. Our results, together with a recent report that GTPγS can stimulate Ca^{2+}-independent secretion of lysosomal enzymes from permeabilized platelets (Athayde and Scrutton, 1990), suggest that secretion of platelet granule constituents is not fundamentally different from exocytosis in cells in which a role for G_E has been postulated.

Results

Effects of Ca^{2+} ions on secretion

We used [^{14}C]5-HT and βTG as markers for the secretion of platelet dense and α-granule constituents, respectively. Permeabilized human platelets contained 71 ± 3 μg of βTG/10^9 cells (mean ± SE, 17 determinations). Incubation of permeabilized platelets for 10 min at 25°C in the absence of added Ca^{2+} ions, or at a buffered pCa of 7, did not cause any release of either [^{14}C]5-HT or βTG. However, incubation with higher Ca^{2+} concentrations (pCa 6 to 4.5) led to secretion from both granule types (Figure 1). There was no consistent difference in the Ca^{2+} sensitivity of the release of [^{14}C]5-HT and βTG, in that the most marked effects of increasing Ca^{2+} concentrations were always obtained at pCa values between 6 and 5. In 10-min incubations, half-maximal secretion from both granule types required a pCa of 5.5 ± 0.1 (mean

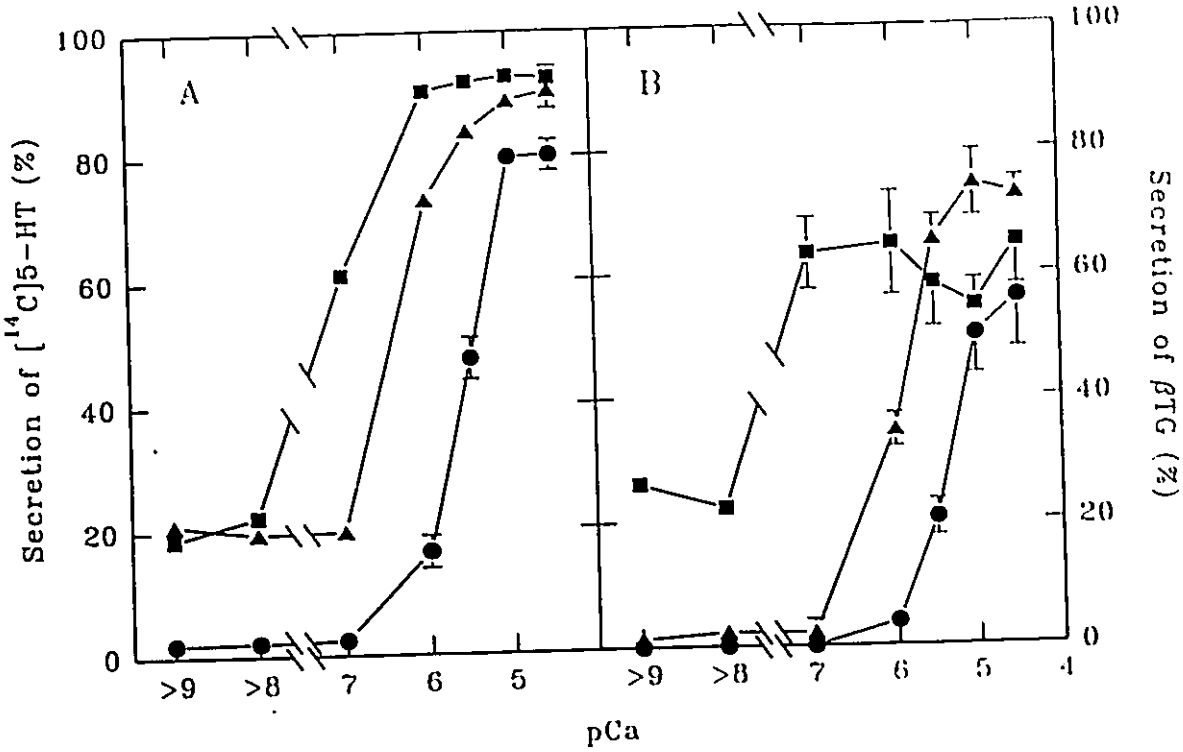

Figure 1. **Effects of PMA and of GTPγS on the secretion of [^{14}C]5-HT (A) and βTG (B) from permeabilized platelets at various buffered Ca^{2+} concentrations.** Samples of permeabilized platelets in a glutamate-based medium containing ATP (see Materials and methods) were equilibrated for 15 min at 0°C with the indicated Ca^{2+} buffers and either no other additions (●), 100 nM PMA (▲), or 100 μM GTPγS (■). The samples were then incubated for 10 min at 25°C and [^{14}C]5-HT and βTG secretion were then determined. Values are means ± SE from three identical incubation mixtures.

± SE, 5 experiments); maximal secretion, observed at a pCa of 4.5, amounted to 81 ± 3% of the platelet [^{14}C]5-HT and 50 ± 3% of platelet βTG (means ± SE). The latter value is significantly less than the former ($p < 0.001$). Higher Ca^{2+} concentrations (e.g., pCa 4) caused less secretion (not shown), probably as a result of Ca^{2+}-dependent proteolysis (Haslam and Davidson, 1984a). Although Ca^{2+} appeared to have very similar effects on the secretion of dense and α-granule constituents, the dose-response curves for βTG release were slightly but consistently steeper than those for [^{14}C]5-HT, as shown by a progressive increase in the ratio of βTG to [^{14}C]5-HT released between pCa values of 6 and 4.5 (Figure 2A). This suggests a minor difference in the regulation of secretion from the two granule types under these conditions. At a pCa of 5, in the absence of other additions, secretion of βTG was only slightly slower than that of [^{14}C]5-HT, with both approaching maximum values after 10–20 min (Figure 3, C and D).

Effects of PMA on secretion

A previous study with permeabilized platelets (Knight and Scrutton, 1984) showed that addition of PMA (16 nM) shifted the Ca^{2+} dose-response curve for secretion of [^{14}C]5-HT to the left and caused just detectable secretion of [^{14}C]5-HT in the absence of added Ca^{2+}. In the present work, we used a higher PMA concentration (100 nM), which was just sufficient to activate PKC optimally in permeabilized platelets (see later), and observed similar though somewhat more marked effects (Figure 1A). Thus, PMA caused 5- to 10-fold increases in the Ca^{2+} sensitivity of [^{14}C]5-HT secretion, significant release of [^{14}C]5-HT in the absence of Ca^{2+} (pCa > 8), and a small but significant increase in the secretion observed at pCa 4.5 ($p < 0.05$). To determine whether or not truly Ca^{2+}-independent release of [^{14}C]5-HT occurs, we also measured secretion in samples containing additional ethylene glycol-bis(β-aminoethyl ether)-*N,N,N',N'*-tetraacetic acid (EGTA, final concentration 12.5 mM, pCa > 9). Under these conditions, the release of [^{14}C]5-HT amounted to 22 ± 1% (mean ± SE, 5 experiments) and was no less than seen with 2.5 mM EGTA (pCa > 8). Thus, we were able to conclude that PMA can induce a significant Ca^{2+}-independent se-

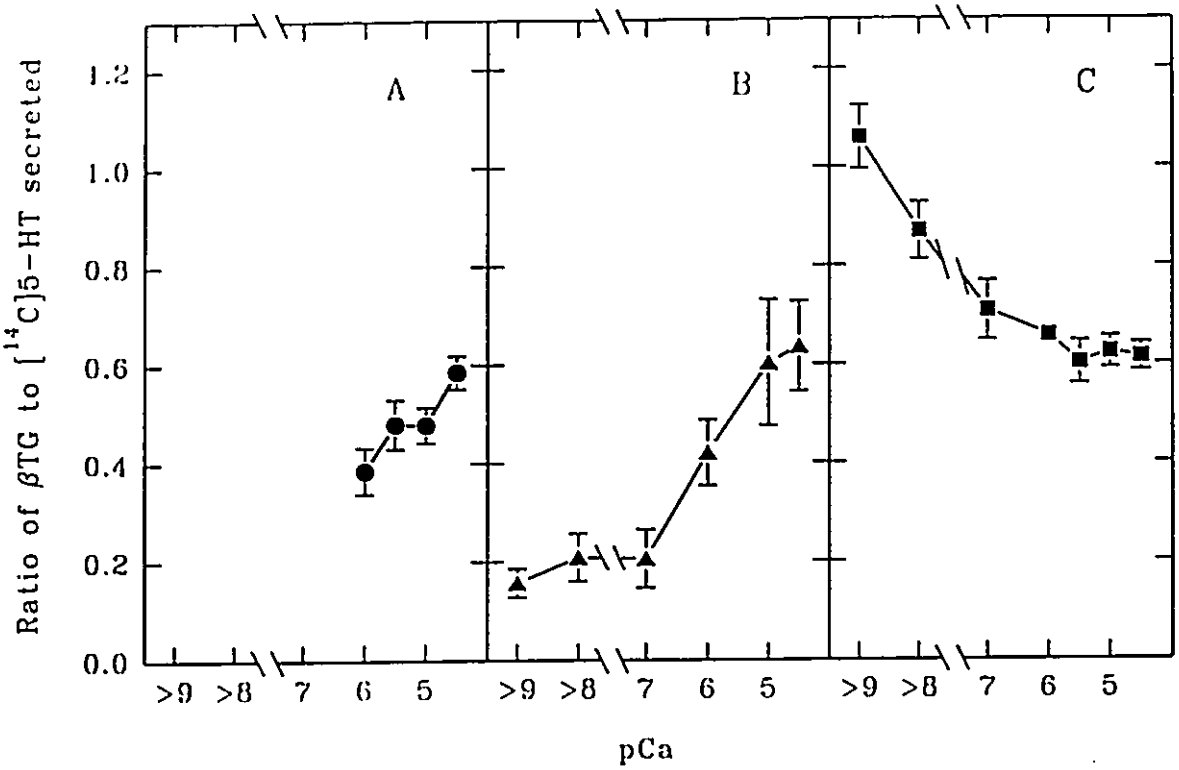

Figure 2. **Effects of GTPγS and of PMA on the relationship between the secretion of βTG and [^{14}C]5-HT from permeabilized platelets at various Ca^{2+} concentrations.** Permeabilized platelets were equilibrated and incubated with the indicated Ca^{2+} buffers and either no other additions (A), 100 nM PMA (B), or 100 μM GTPγS (C), as described for Figure 1. The percent secretion of βTG and of [^{14}C]5-HT were determined for each condition and expressed as a ratio. Values are means ± SE from 3 to 11 separate experiments. When secretion of βTG and [^{14}C]5-HT was negligible (A, pCa > 6), ratios were not calculated.

cretion of [^{14}C]5-HT from permeabilized platelets. Analysis of the time-course of [^{14}C]5-HT release showed that, at a pCa > 9, dense granule secretion continued for at least 20 min in the presence of PMA (Figure 3A). At a pCa of 5, secretion was much more rapid and, in the presence of PMA, was almost complete within 5 min (Figure 3C).

As observed for the secretion of [^{14}C]5-HT, incubation of permeabilized platelets with 100 nM PMA enhanced the Ca^{2+}-sensitivity of secretion of βTG and increased the maximum extent of secretion observed at a pCa of 4.5 (Figure 1B). However, 100 nM PMA caused very little secretion of βTG in the absence of Ca^{2+} ions. Thus, at a pCa > 9, the platelet βTG found in the supernatant after incubation for 10 min with 100 nM PMA amounted to only 3 ± 1% (mean ± SE, 5 experiments), and the ratio of βTG to [^{14}C]5-HT released (calculated as percentages of the platelet content) was only 0.16 ± 0.03 (mean ± SE). This ratio increased progressively as the Ca^{2+} concentration was increased from a pCa of 7 to 4.5, reaching a value of 0.64 ± 0.09 (Figure 2B), essentially the same as observed in the absence of PMA. In the presence of PMA, secretion of βTG was slightly delayed relative to that of [^{14}C]5-HT (Figure 3, C and D). These results imply a significant difference between the regulatory mechanisms responsible for the secretion of dense and α-granule constituents.

Effects of guanine nucleotides on secretion

Previous work with electropermeabilized platelets prepared as in the present study showed that 100 μM GTPγS greatly enhanced the Ca^{2+} sensitivity of the secretion of [^{14}C]5-HT and also caused some secretion in the absence of added Ca^{2+} (Haslam and Davidson, 1984b,c); small amounts of Ca^{2+} in the reagents or released from the platelets appeared to be partly responsible for the latter effect. We have now reinvestigated these findings and have compared [^{14}C]5-HT release with that of βTG (Figure 1).

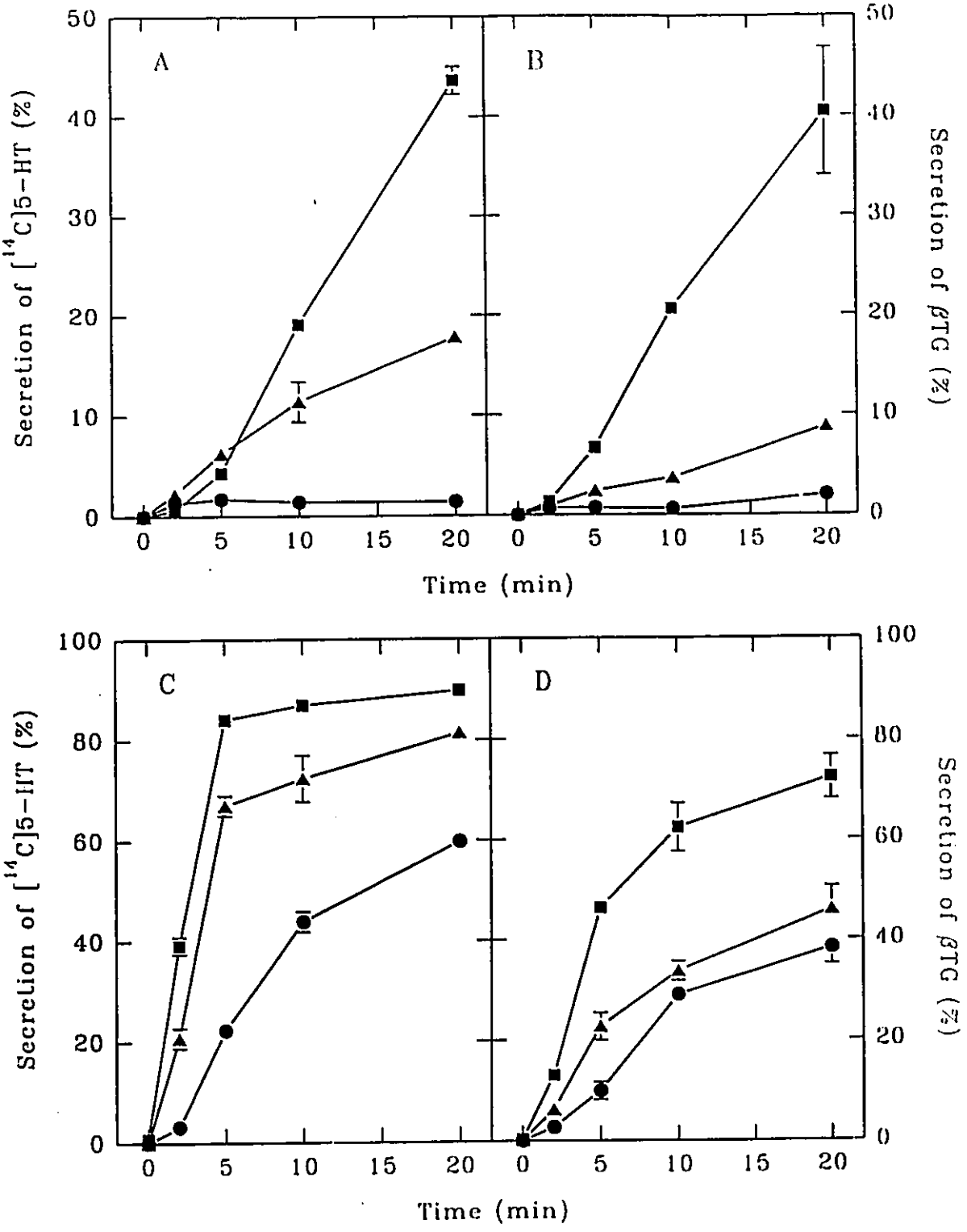

Figure 3. **Effects of PMA and of GTPγS on the time course of secretion of [^{14}C]5-HT (A and C) and βTG (B and D) from permeabilized platelets.** Experiments were carried out as for Figure 1 at pCa values of either >9 (A and B) or 5 (C and D); the period of incubation at 25°C was varied as indicated. Samples were equilibrated and incubated with no other additions (●), with 100 nM PMA (▲), or with 100 μM GTPγS (■). Values are means ± SE from three identical incubation mixtures.

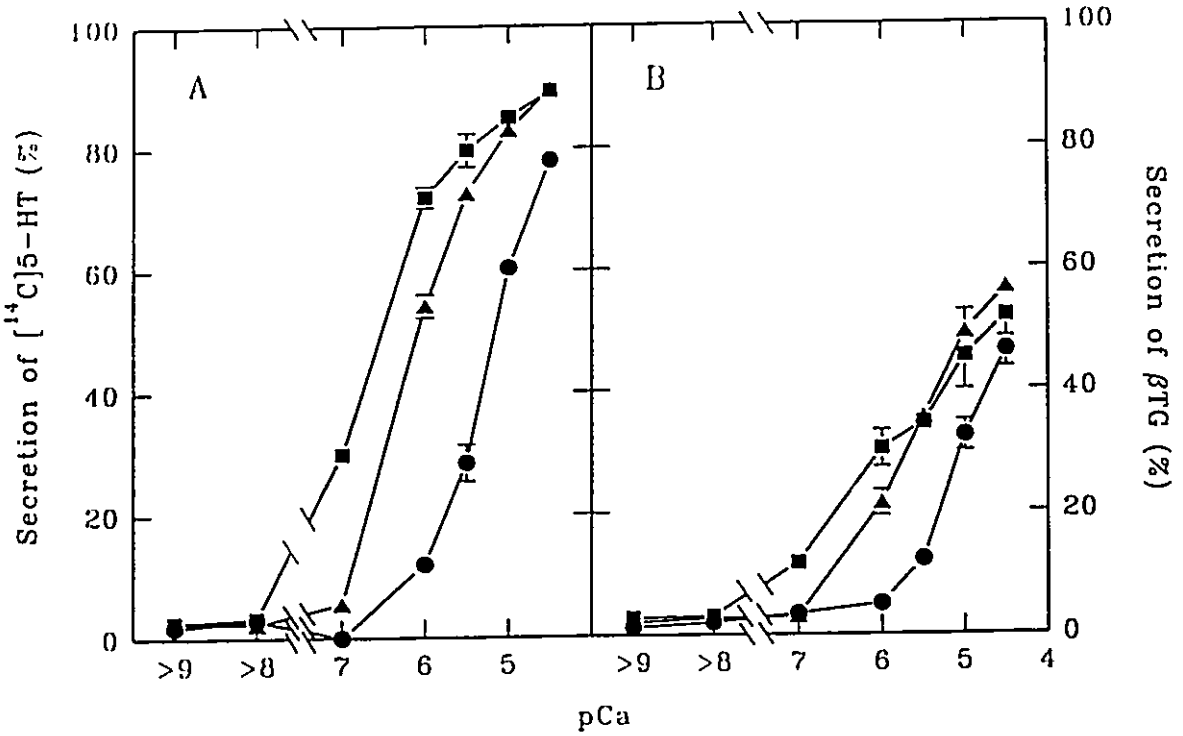

Figure 4. **Effects of GTP and of GTPγS on the secretion of [^{14}C]5-HT (A) and βTG (B) from permeabilized platelets at various buffered Ca^{2+} concentrations.** Samples of permeabilized platelet suspension were equilibrated for 15 min at 0°C with the indicated Ca^{2+} buffers and either no other additions (●), 100 μM GTP (▲), or 10 μM GTPγS (■). Samples were then incubated for 10 min at 25°C and [^{14}C]5-HT and βTG secretion were then determined. Values are means ± SE from three identical incubations.

We found that 100 μM GTPγS caused substantial secretion from both granule types in the presence of 12.5 mM EGTA (pCa > 9), amounting to 25 ± 2% of the platelet [^{14}C]5-HT and 26 ± 2% of platelet βTG (means ± SE, 10 experiments). These values were only slightly less than ([^{14}C]5-HT) or the same as (βTG) those obtained with 2.5 mM EGTA (pCa > 8). Thus, 100 μM GTPγS differed in its effects from 100 nM PMA, which induced Ca^{2+}-independent secretion mainly from the dense granules. GTPγS also enhanced the maximal secretion of [^{14}C]5-HT and of βTG, again observed at a pCa of 4.5, to values of 92 ± 3% and 57 ± 5%, respectively (means ± SE; 4 experiments). These results show that in the absence of Ca^{2+} ions, 100 μM GTPγS induced the secretion of a much higher percentage of the releasable βTG (49 ± 4%) than of the releasable [^{14}C]5-HT (27 ± 4%) (means ± SE, 4 experiments), indicating that the actions of GTPγS favor the secretion of α-granule constituents under these conditions. As the Ca^{2+} concentration was increased in the presence of 100 μM GTPγS, the ratio of βTG to [^{14}C]5-HT released decreased progressively to reach a value similar to that seen in the absence of GTPγS (Figure 2C). In the absence of Ca^{2+} ions, the GTPγS-induced secretion of both [^{14}C]5-HT and βTG proceeded slowly and almost linearly for 20 min, apart from an initial 2-min lag (Figure 3, A and B), whereas at a pCa of 5, secretion of both granule constituents was greatly accelerated, with that of [^{14}C]5-HT slightly preceding that of βTG, as also observed with PMA (Figure 3, C and D).

Ca^{2+}-independent secretion of [^{14}C]5-HT and βTG was not observed in the presence of either 10 μM GTPγS or 100 μM GTP. However, these additions enhanced the Ca^{2+}-sensitivity of secretion of both [^{14}C]5-HT and βTG and, to some extent, the maximum secretion obtained (Figure 4). As reported previously for [^{14}C]5-HT (Haslam and Davidson, 1984b), 10 μM GTPγS shifted the Ca^{2+} dose-response curve for βTG secretion further to the left than did 100 μM GTP and even promoted limited secretion at a pCa of 7. Thus, in the pCa range in which these concentrations of guanine nucleotides were effective (7 to 4.5), no significant dissociation of [^{14}C]5-HT and βTG secretion was apparent.

Effects of thrombin on secretion

Addition of an optimal dose of thrombin (2 U/ml; Haslam and Davidson, 1984a) increased the Ca^{2+}-sensitivity of secretion from both dense and α-granules to similar extents, but caused no release of [^{14}C]5-HT or βTG in the absence of Ca^{2+} ions (Figure 5). Thrombin also increased the maximum secretion of βTG and, to a lesser extent, of [^{14}C]5-HT. The effects of this concentration of thrombin (Figure 5) were quantitatively very similar to those of 100 μM GTP (Figure 4). Addition of a low GTP concentration (10 μM) with thrombin caused a further shift to the left of the Ca^{2+} dose-response curves for secretion, so that substantial effects were seen at a pCa of 7 (as previously reported for [^{14}C]5-HT by Haslam and Davidson, 1984b). However, no secretion of either granule constituent was detected in the complete absence of Ca^{2+} ions (Figure 5). These results suggest that addition of thrombin, or of thrombin with GTP, stimulated secretion of both dense and α-granule constituents by the same or similar Ca^{2+}-dependent mechanisms.

Synergistic effects of PMA and GTPγS on secretion

The ability of GTPγS to induce secretion of βTG from permeabilized platelets in the absence of Ca^{2+} ions, a condition in which PMA had little effect, suggested that the action of the former compound might not be fully accounted for by the activation of PKC. We therefore studied the effects of increasing concentrations of GTPγS on Ca^{2+}-independent secretion in the absence and presence of an optimal concentration of PMA (100 nM). In 10-min incubations in the absence of PMA, the release of platelet [^{14}C]5-HT and βTG caused by GTPγS did not exceed 40%, even with 1 mM of the latter compound (Figure 6, A and B). Secretion of [^{14}C]5-HT and βTG caused by 100 μM GTPγS amounted to 70–80% of the maximum observed with 1 mM GTPγS in the absence of Ca^{2+}. However, when PMA was included in the incubation mixtures, the effects of all concentrations of GTPγS on the release of both [^{14}C]5-HT and βTG were enhanced (Figure 6, A and B). This effect was particularly striking with 10 μM GTPγS, which did not cause

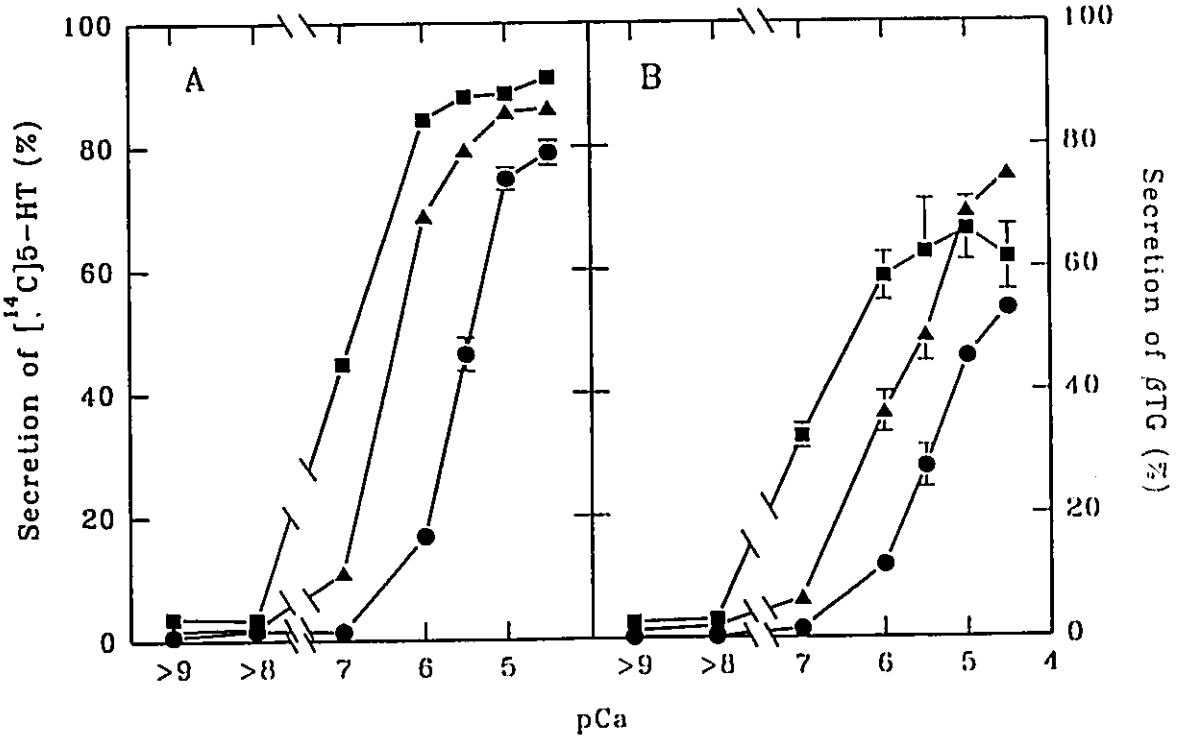

Figure 5. **Effects of GTP and thrombin on the sensitivity to Ca^{2+} of the secretion of [^{14}C]5-HT (A) and βTG (B) from permeabilized platelets.** Samples of permeabilized platelet suspension were equilibrated for 15 min at 0°C with the indicated Ca^{2+} buffers and were then incubated for 10 min at 25°C. Samples contained either no other additions (●) or 2 U of thrombin/ml added after equilibration with Ca^{2+} alone (▲) or with Ca^{2+} and 10 μM GTP (■). Secretion of both [^{14}C]5-HT and βTG were then determined. Values are means ± SE from three identical incubation mixtures.

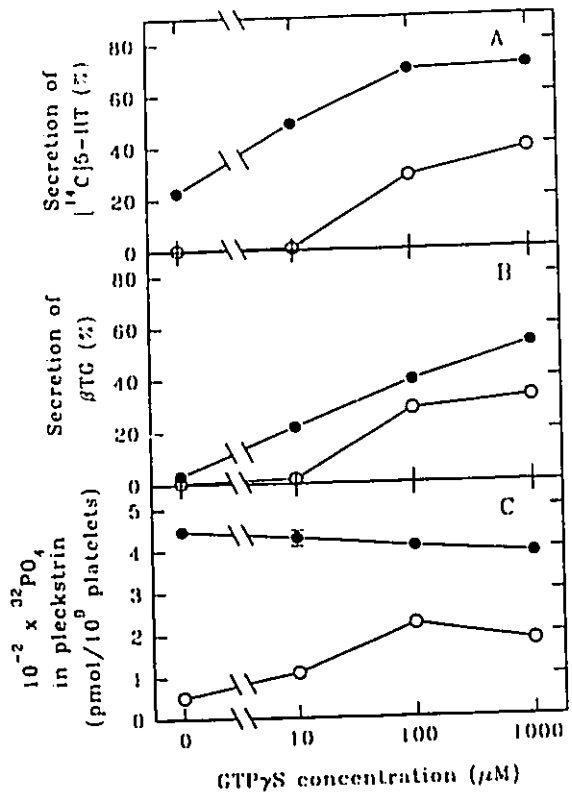

Figure 6. **Ca^{2+}-independent secretion of [^{14}C]5-HT and βTG from permeabilized platelets by different concentrations of GTPγS added without or with PMA; relationship to pleckstrin phosphorylation.** Samples of permeabilized platelets containing 12.5 mM EGTA (pCa > 9) were equilibrated for 15 min at 0°C with the indicated concentrations of GTPγS in the absence (○) or presence (●) of 100 nM PMA and were then incubated for 10 min at 25°C. The secretion of [^{14}C]5-HT (A) and βTG (B) were measured in three identical samples; mean values ± SE are shown. Parallel duplicate incubations were carried out with permeabilized platelet suspension containing [γ-^{32}P]ATP for measurement of the phosphorylation of pleckstrin (C); mean values ± range are shown.

any secretion by itself. These supra-additive effects of GTPγS and an optimal concentration of PMA provide further evidence that GTPγS must exert its effects, at least in part, by a mechanism other than activation of PKC.

Relationship between protein phosphorylation and secretion

The phosphorylation of pleckstrin, the major substrate of PKC in platelets (Imaoka *et al.*, 1983; Sano *et al.*, 1983; Tyers *et al.*, 1988), was used as an index of the extent to which this enzyme was activated during secretion. As expected, incubation of permeabilized platelets with 100 nM PMA in the presence of [γ-^{32}P]ATP caused a marked increase in the ^{32}P-labeling of pleckstrin (Figures 6C and 7A). In the absence of Ca^{2+} ions, phosphorylation of pleckstrin amounted to 481 ± 28 pmol/10^9 platelets (mean ± SE, 3 experiments) and was not further increased by addition of GTPγS (Figure 6C) or Ca^{2+} ions (Figure 7A). Indeed, both GTPγS and high Ca^{2+} concentrations tended to decrease the phosphorylation of pleckstrin seen in the presence of PMA. Lower concentrations of PMA (20 and 50 nM) caused only slightly less phosphorylation than 100 nM (not shown), indicating that 100 nM was optimal. Addition of GTPγS alone in the absence of Ca^{2+} ions (pCa > 9) also caused a phosphorylation of pleckstrin amounting to 53 ± 2% of the maximum extent seen with PMA (mean ± SE, 3 experiments). Addition of Ca^{2+} ions slightly increased the phosphorylation of pleckstrin observed in the presence of GTPγS, but never to the level seen with PMA alone (Figure 7A).

As reported previously (Haslam and Davidson, 1984a), addition of Ca^{2+} ions alone to permeabilized platelets increased the phosphorylation of both pleckstrin and myosin light chain (Figure 7). Although phosphorylation of pleckstrin was optimal at a pCa of 4.5, reaching a level similar to that seen in the presence of GTPγS and Ca^{2+}, this reaction did not correlate well with secretion, in that substantial phosphorylation was seen at a pCa value as low as 6. Although PMA and, to a lesser extent, GTPγS enhanced the phosphorylation of myosin light chain at pCa values in the range >9–5, presumably as a result of the activation of PKC, the dominant factor regulating the phosphorylation of this protein was the Ca^{2+} concentration (Figure 7B). This contrasts with the largely Ca^{2+}-independent effects of PMA and GTPγS on the phosphorylation of pleckstrin (Figure 7A).

Activation of phosphoinositide-specific phospholipase C

Previous studies in this laboratory have shown that GTPγS stimulates the release of [^{3}H]inositol phosphates from [^{3}H]phosphoinositides by phospholipase C in permeabilized platelets incubated at a pCa of 7 and that this effect was much diminished in the absence of added Ca^{2+} ions (pCa > 8) (Culty *et al.*, 1988). We have now shown that no significant [^{3}H]inositol phosphate formation could be detected in the presence or absence of 100 μM GTPγS, when the Ca^{2+} ion concentration was further reduced by additional EGTA (pCa > 9) (Table 1). This finding suggests

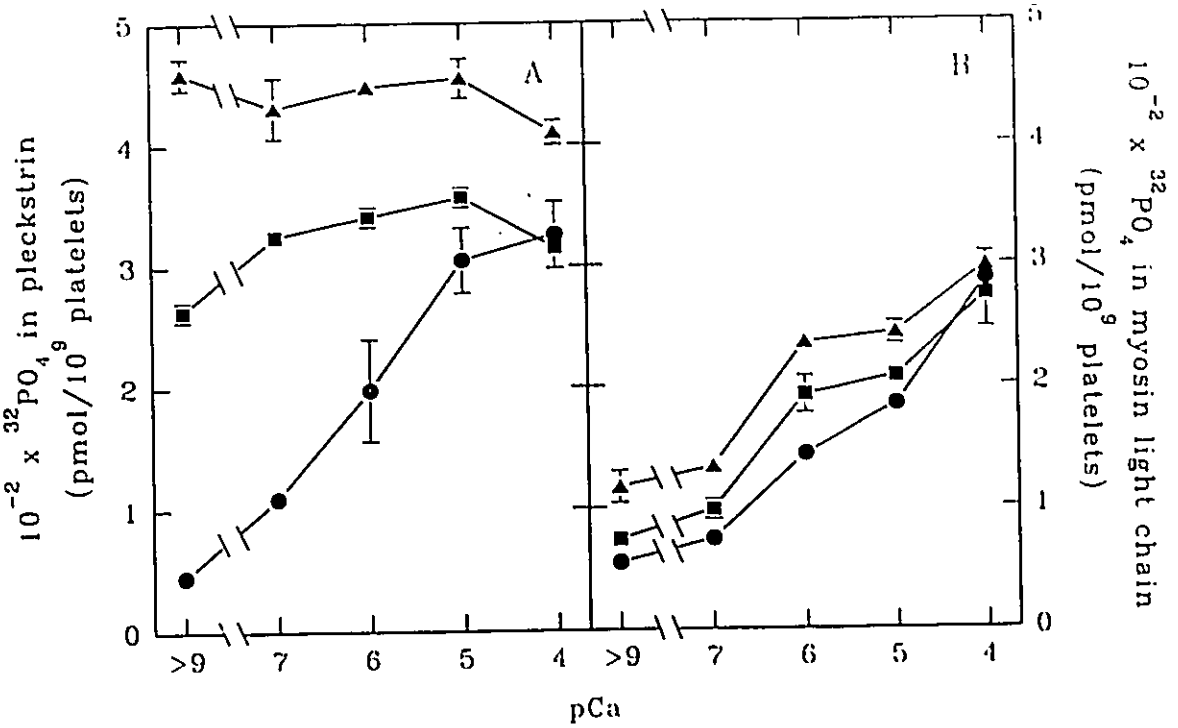

Figure 7. **Effects of Ca^{2+}, PMA, and GTPγS on the phosphorylation of pleckstrin (A) and myosin light chain (B) in permeabilized platelets.** Samples of permeabilized platelet suspension containing [γ-^{32}P]ATP were equilibrated for 15 min at 0°C with the indicated Ca^{2+} buffers and either no other additions (●), 100 nM PMA (▲), or 100 μM GTPγS (■). The samples were then incubated for 10 min at 25°C before the addition of trichloroacetic acid and analysis of ^{32}P-labeled proteins by sodium dodecyl sulfate–polyacrylamide gel electrophoresis. Areas of gels containing pleckstrin and myosin light chain were cut out and counted for ^{32}P. Values are means ± range from duplicate incubation mixtures.

that the Ca^{2+}-independent activation of PKC by GTPγS was not mediated by DAG released from phosphoinositides. This conclusion is supported by the finding that, although there was trace formation of [^{3}H]inositol phosphates at pCa 7 in the absence of GTPγS (Table 1), the associated phosphorylation of pleckstrin (Figure 7A) was much less than observed with GTPγS at a pCa > 9, when no phosphoinositide-specific phospholipase C activity was detected. The enhanced Ca^{2+}-independent secretion of 5-HT and βTG caused by addition of GTPγS in the presence of PMA (Figure 6) was also not associated with any [^{3}H]inositol phosphate formation; indeed, PMA inhibited the stimulation of [^{3}H]-inositol phosphate formation caused by GTPγS at a pCa of 7 (Table 1).

Discussion

We selected βTG as the most appropriate marker for α-granule secretion because many of the others, including platelet factor 4, bind extensively to the platelet surface (Stenberg *et al.*, 1984; Capitanio *et al.*, 1985), usually by Ca^{2+}-dependent mechanisms (George and Onofre, 1982). Our results show that secretion of βTG from electropermeabilized platelets, as monitored by release of βTG into the platelet supernatant, was stimulated by the addition of Ca^{2+} ions in a manner very similar to the secretion of dense granule 5-HT. Thus, half-maximal secretion was seen at a pCa of 5.5 in both instances. However, of the total βTG in permeabilized platelets (~70 μg/10^9 platelets), only 50–60% could be released at an optimal Ca^{2+} concentration (pCa 4.5), the exact value depending on whether or not a second stimulus was present. The Ca^{2+}-stimulated release of an even lower percentage of platelet factor 4 from permeabilized platelets has recently been reported (Peltola and Scrutton, 1990). This maximum secretion of βTG should be compared with the much more complete release of dense granule 5-HT (80–90%). However, the βTG released in our experiments was similar to the maximum secreted from intact platelets in response to thrombin (38 μg/10^9 platelets; Akkerman *et al.*,

Table 1. Effects of GTPγS and PMA on total [^{3}H]inositol phosphate formation in permeabilized platelets incubated in the presence and absence of Ca^{2+} ions

Additions	[^{3}H]inositol phosphates (dpm/10^9 platelets)	
	pCa > 9	pCa 7
None	−250 ± 187	508 ± 211
PMA (100 nM)	−166 ± 183	129 ± 211
GTPγS (100 μM)	31 ± 192	11 008 ± 434
GTPγS (100 μM) + PMA (100 nM)	−15 ± 197	4 894 ± 247

Platelets labeled with both [^{3}H]inositol and [^{14}C]5-HT were permeabilized, as described under Materials and methods. Samples were equilibrated for 15 min at 0°C with the additions indicated at the designated pCa values and were then incubated for 10 min at 25°C before extraction and measurement of [^{3}H]inositol phosphates. Amounts found in the suspension of permeabilized platelets before equilibration of the samples (1333 ± 182 dpm/10^9 platelets) were subtracted. Values given are means ± SE of the difference from three identical samples. The secretion of [^{14}C]5-HT at a pCa > 9 (not shown) was similar to that illustrated in Figure 6.

1982) or released from platelets by physical methods of disruption (Rink *et al.*, 1983; Krishnamurthi *et al.*, 1986; Coorssen and Haslam, unpublished). This suggests that platelets contain a form of βTG that is not readily secreted. Preliminary experiments suggest that this βTG is membrane-bound, because it is not released by freezing and thawing followed by sonication, but can then be extracted partly by 2 M KCl, as well as by detergent (Coorssen and Haslam, unpublished). It is possible that this pool of βTG is the source of that found on the plasma membrane after stimulation of platelets with thrombin (Stenberg *et al.*, 1984).

Incubation of permeabilized platelets with thrombin, particularly in the presence of GTP, with low concentrations of GTPγS (e.g., 10 μM), or with PMA, all shifted the Ca^{2+} dose-response curve for the release of βTG to the left, either into or beyond the physiological range (pCa 7 to 6), as previously reported for dense granule 5-HT (Haslam and Davidson, 1984a,b; Knight and Scrutton, 1984, 1986). Thus, the regulation of the secretion of α-granule constituents from permeabilized platelets resembles much more closely that of dense granule constituents than that of lysosomal hydrolases, since in the latter case, the above stimuli enhance the maximum response to Ca^{2+} rather than the Ca^{2+} sensitivity of secretion (Knight *et al.*, 1984; Athayde and Scrutton, 1990). Because thrombin and guanine nucleotides activate phosphoinositide-specific phospholipase C in permeabilized platelets (Brass *et al.*, 1986; Culty *et al.*, 1988), enhance the formation of DAG (Haslam and Davidson, 1984a,c; Brass *et al.*, 1986), and promote the phosphorylation in these cells of the PKC substrate pleckstrin (Haslam and Davidson, 1984a; Figure 7), the similar effects of these stimuli and of PMA on secretion from permeabilized platelets can plausibly be explained by the activation of PKC. However, high concentrations of GTPγS (e.g., 100 μM) were far more effective in promoting the release of 5-HT and particularly of βTG than was 100 nM PMA, which stimulated the phosphorylation of pleckstrin to the maximum extent possible in permeabilized platelets and always to a higher level than was observed with GTPγS. Moreover, although Ca^{2+} enhanced the phosphorylation of pleckstrin, presumably by activating PKC, this effect did not correlate well with secretion in either a previous study (Haslam and Davidson, 1984a) or the present work. This is not to say that PKC is unimportant. The potentiating effects of PMA and synthetic DAG (Knight and Scrutton, 1984; Athayde and Scrutton, 1990) and preliminary results showing that complete inhibition of PKC markedly reduces but does not abolish the effects of GTPγS and Ca^{2+} on secretion (Haslam and Davidson, 1990) both indicate that PKC plays a major role in secretion of platelet granule constituents. It is, nevertheless, clear that both GTPγS and Ca^{2+} ions promote secretion of dense and α-granule constituents by mechanisms additional to the activation of PKC.

Thus, as in other cells (Gomperts, 1990), there appear to be three major factors involved in the regulation of secretion from platelets, namely Ca^{2+} ions, guanine nucleotide, and PKC. Our results indicate that these factors exert independent as well as interacting effects with significantly different consequences for the secretion of dense and α-granule constituents. Since the ratio of the maximum percentages of βTG and 5-HT secreted was ~0.6 (at pCa 4.5), whether or not PMA or GTPγS was present (Figure 2), departures from this ratio under conditions of suboptimal stimulation are indicative of selective secretion from one or the other granule type. By this criterion, low Ca^{2+} concentrations (pCa 7 to 6) tended to favor secretion from dense granules when either no other stimulus or PMA was present, but induced the parallel secretion of both dense and α-granule constituents when GTPγS was present. However, the clearest dissociation of dense and α-granule secretion was obtained in the effective absence of Ca^{2+} ions. When no $CaCl_2$ was added to the

permeabilized platelets, only limiting pCa values could be calculated, since the Ca^{2+} present depended on reagent contamination and the extent to which platelet Ca^{2+} was released into the medium. However, even with the most conservative assumptions (see Materials and methods), pCa values in incubation mixtures (pH 7.4) containing no $CaCl_2$ and either 2.5 or 12.5 mM EGTA were >8 or >9, respectively. Because little difference in secretion was observed under these two conditions with any stimulus (see Figure 1), we believe that such effects can reasonably be described as Ca^{2+}-independent. In particular, PMA induced the slow Ca^{2+}-independent secretion of dense granule 5-HT, as has also been found by others (Knight and Scrutton, 1984; Athayde and Scrutton, 1990). However, PMA caused little release of βTG under these conditions, suggesting that activation of PKC is an insufficient stimulus for α-granule secretion. In intact platelets, PMA has been reported to stimulate the secretion of both dense and α-granule constituents (Rink *et al.*, 1983; Krishnamurthi *et al.*, 1986). The reason for this discrepancy is not known, but it is possible that additional factors play a role in the intact system. In the absence of Ca^{2+}, 100 μM GTPγS differed markedly from 100 nM PMA in its effects on secretion from permeabilized platelets, causing release of both βTG and 5-HT, but preferentially of the former (ratio over 1.0, Figure 2C). Selective secretion of platelet factor 4 in response to GTPγS has also recently been noted (Peltola and Scrutton, 1990). Our results also showed that GTPγS stimulated the phosphorylation of pleckstrin in the absence of Ca^{2+}, but much less effectively than PMA. This again indicates that GTPγS has effects, particularly on the secretion of βTG, that cannot be accounted for by the activation of PKC, a conclusion that is further strengthened by the enhanced stimulation of secretion by GTPγS in the presence of an optimal concentration of PMA. Synergism between GTPγS and PMA in the absence of Ca^{2+} was also observed with respect to the secretion of β-glucuronidase from permeabilized HL60 cells (Stutchfield and Cockcroft, 1988).

The differences that we have observed between the effects of PMA and GTPγS are not sufficient to establish that the latter acts through a GTP-binding protein coupled to an effector other than phosphoinositide-specific phospholipase C, because the diacylglycerol formed by this enzyme could well potentiate secretion through effects additional to the activation of PKC. It is, therefore, crucial that we have been able to show for the first time in platelets that GTPγS induces secretion under conditions in which there is no detectable phosphoinositide breakdown. In this regard, our results are similar to those obtained in experiments with permeabilized adrenal chromaffin cells (Bittner *et al.*, 1986) and RINm5F cells (Vallar *et al.*, 1987). Secretion has also been dissociated from phospholipase C activity by neomycin in streptolysin O-permeabilized mast cells (Cockcroft *et al.*, 1987), studies of which have provided the best evidence that a specific GTP-binding protein, G_E, mediates exocytosis (Gomperts, 1986, 1990). Although secretion from permeabilized mast cells required the simultaneous presence of Ca^{2+} and GTPγS, when the principal anion present was Cl^- (Howell *et al.*, 1987), these stimuli exerted independent effects very similar to those we obtained in platelets, when the anion was glutamate (Churcher and Gomperts, 1990). Thus, there may be no essential difference between exocytosis from platelets and from cells in which a role for a hypothetical G_E has been postulated.

Although the mechanism by which GTPγS stimulates secretion from permeabilized platelets in the absence of Ca^{2+} ions remains obscure, it is associated with the phosphorylation of pleckstrin. The function of pleckstrin is still unknown (Tyers *et al.*, 1988), but phosphorylation of this protein does indicate that activation of PKC has occurred (Sano *et al.*, 1983). Platelets have, in fact, been shown to contain an unidentified isozyme of PKC that can phosphorylate pleckstrin in the absence of Ca^{2+} (Tsukuda *et al.*, 1988), and current results show that PKC is essential for the Ca^{2+}-independent secretion of dense and α-granule constituents, caused by GTPγS (Coorssen, Davidson, and Haslam, unpublished). These findings imply that, even in the absence of Ca^{2+}, GTPγS acts on a G-protein–coupled effector enzyme that generates a lipid second messenger capable of stimulating PKC. Because GTPγS also has an essential action that is not accounted for by activation of PKC, it is possible that a product of the same effector enzyme stimulates secretion by another mechanism, perhaps by acting as a membrane fusogen. Alternatively, GTPγS may stimulate Ca^{2+}-independent secretion through two different GTP-binding proteins. Candidate effector enzymes that could be involved in the formation of activators of PKC and possibly of fusogenic lipids in the absence of Ca^{2+} include a form of phospholipase C that utilizes substrates other than the phosphoinositides, phospholipase A_2 and phospholipase D. A phosphatidylcholine

phospholipase C that is Ca^{2+} independent and is activated by GTPγS has been described in liver membranes (Irving and Exton, 1987) but has not so far been reported in platelets. A GTPγS-activated phospholipase A_2 is present in platelet membranes but appears to require Ca^{2+} (Akiba *et al.*, 1989; Silk *et al.*, 1989). Finally, we have recently shown that rabbit platelet membranes contain a phospholipase D activity that is stimulated by GTPγS in the absence of Ca^{2+}, though this ion does enhance enzyme activity (Haslam *et al.*, 1990; Van der Meulen and Haslam, 1990). In all cases, PMA treatment appears to potentiate the actions of GTPγS on these enzymes, which would be consistent with the effect of PMA on GTPγS-induced secretion from permeabilized platelets. Each of these potential effector enzymes can generate, directly or indirectly, DAG and/or arachidonate, both of which can activate platelet PKC in the absence of Ca^{2+} (Tsukuda *et al.*, 1988). Moreover, DAG (Siegel *et al.*, 1989; Coorssen and Rand, 1990), arachidonate (Meers *et al.*, 1988), and the immediate product of phospholipase D action, phosphatidate (Sundler and Papahadjopoulos, 1981; Leventis *et al.*, 1986), are all potentially fusogenic. However, exogenous phospholipase C does not stimulate secretion from permeabilized adrenal chromaffin cells, despite substantial DAG formation (Eberhard *et al.*, 1990). Moreover, secretion has been dissociated from phospholipase A_2 activation in permeabilized mast cells (Churcher *et al.*, 1990). For these reasons, activation of phospholipase D may be the most promising candidate. A role for phospholipase D–generated phosphatidate or its metabolites in exocytosis from mast cells has recently been proposed (Gruchalla *et al.*, 1990), and it may be significant that activation of this enzyme is particularly prominent in neutrophils (Billah *et al.*, 1989), the cell in which a phospholipase C–independent effect of GTPγS on secretion was first detected (Barrowman *et al.*, 1986).

Materials and methods

Materials

Radioimmunoassay (RIA) kits for βTG, [*side-chain*-2-^{14}C]5-HT (55 mCi/mmol) and ACS scintillant were from Amersham (Oakville, Ont.). [γ-^{32}P]ATP was from Du Pont Canada (Mississauga, Ont.). GTPγS was obtained from Boehringer Mannheim Canada (Laval, Que.). ATP (prepared by phosphorylation of adenosine), GTP, PMA, piperazine-*N*,*N*'-bis(2-ethanesulfonic acid) (PIPES), *N*-2-hydroxyethylpiperazine-*N*'-2-ethanesulfonic acid (HEPES), L-glutamic acid, and bovine serum albumin (fraction V) were from Sigma (St. Louis, MO). Human α-thrombin (2700 U/mg) was kindly provided by Dr. J.W. Fenton II of the New York State Department of Health (Albany, NY). Other materials were from sources listed in Haslam and Davidson (1984a) and Colty *et al.* (1988).

Labeling and permeabilization of human platelet suspensions

Human platelets were isolated, labeled with [^{14}C]5-HT, and washed, as described in Haslam and Davidson (1984a). Labeled platelets—in Ca^{2+}-free Tyrode's solution (pH 6.5) containing 5 mM PIPES, 0.35% (w/v) bovine serum albumin, and 5 mM EGTA—were electropermeabilized in a 0.2-cm-wide chamber by 10 discharges from 4.5-μF capacitors charged at 3 kV; this procedure yielded permeabilized platelets with a sensitivity to Ca^{2+} identical to that described by Haslam and Davidson (1984a). As previously, the permeabilized platelets were cooled to 4°C, applied to a column of Sepharose CL-4B, and eluted with a medium (pH 7.4) containing 12.5 mM $MgCl_2$ and the potassium salts of glutamic acid (160 mM), HEPES (20 mM), EGTA (2.5 mM), and EDTA (2.5 mM) (buffer A). Immediately after elution, the platelet suspension was diluted with buffer A containing ATP to give 3–5 × 10^8 platelets/ml (final ATP, 5 mM) and stored at 0°C until used (within 2 h).

Incubations

Samples (160 μl) of permeabilized platelet suspension were mixed with 40 μl of buffer A containing any guanine nucleotide and the $CaCl_2$ required to give a particular pCa value (Haslam and Davidson, 1984b). PMA or vehicle (0.4 μl of dimethylsulfoxide) was added at the same time as the platelets to prevent the PMA from coming out of solution. These mixtures were equilibrated for 15 min at 0°C before transfer to 25°C and further incubation for up to 20 min. Thrombin, if present, was added in 2 μl of buffer A at the time of transfer to 25°C. Incubations were terminated by addition of 200 μl of ice-cold buffer (pH 7.4) containing EDTA (35 mM), glutamate (80 mM), and HEPES (20 mM) and immediate centrifugation at 12 000 × *g* for 1 min. Supernatants were quickly recovered for determination of the platelet [^{14}C]5-HT and βTG released.

The concentration of $CaCl_2$ required to give the desired pCa values in the EGTA/EDTA/$MgCl_2$ buffer system used were calculated according to Fabiato and Fabiato (1979). The pH values of the $CaCl_2$ solutions were adjusted so that a final pH of 7.4 was obtained after mixing with the other additions. The $[Ca^{2+}]_{free}$ in incubation mixtures containing no added $CaCl_2$ cannot be determined accurately, but, assuming that the total platelet Ca^{2+} (80 nmol/mg protein; Feinstein and Fraser, 1975) was released into the medium during incubations and that reagent contamination cannot exceed 20 μM Ca^{2+}, we calculate that the pCa value under these conditions must be >8. To verify that this $[Ca^{2+}]_{free}$ has no functional effect, we increased the EGTA concentration in some incubation mixtures from 2.5 to 12.5 mM, which decreases the $[Ca^{2+}]_{free}$ sixfold and, on the above assumptions, gives a pCa value >9 in the absence of added $CaCl_2$.

Measurement of secretion from dense and α-granules

Release of platelet [^{14}C]5-HT was measured by counting 200 μl of supernatant in ACS scintillant; βTG in the supernatant was determined by RIA. Total platelet [^{14}C]5-HT and βTG were determined in permeabilized platelets lyzed by addition of 0.01 vol. of 10% (w/v) Triton X-100. The secretion of each of these granule constituents was expressed as a percentage of the total present in the permeabilized platelet suspension after subtraction of that found in supernatant

from permeabilized platelets that had not undergone equilibration or incubation. The latter amount was never more than 5% of the total [^{14}C]5-HT and 1% of the total βTG in the permeabilized platelets.

The RIA procedure described by Amersham was modified to permit 250 assays/kit. The assay volume was reduced from 450 to 90 μl, comprising 40 μl of ^{125}I-labeled βTG, 40 μl of anti-βTG serum, and 10 μl of βTG standard or of a dilution of platelet supernatant or lysate. Centrifugation of the $(NH_4)_2SO_4$-precipitated antigen-antibody complex was carried out at 12 000 × *g* for 2 min, and the pellet was then washed with an equivalent amount of $(NH_4)_2SO_4$ solution. Both βTG standards and dilutions of experimental samples were adjusted to contain 0.001% Triton X-100. Failure to include Triton X-100 in assays of supernatant samples reduced the values of βTG obtained.

Phosphorylation of platelet proteins

To measure protein phosphorylation in permeabilized platelets, the methods of Haslam and Davidson (1984a) were used. Suspensions of permeabilized platelets (containing 5 mM ATP) were mixed with [γ-^{32}P]ATP to give 100 μCi/ml. Samples (final volume 0.2 ml) were equilibrated and incubated with appropriate additions, as described above, and incubations were then terminated by addition of 1.0 ml of 10% (w/v) trichloroacetic acid. Platelet protein was analyzed by sodium dodecyl sulfate–polyacrylamide gel electrophoresis (Imoaka *et al.*, 1983). After autoradiography, the regions of the gel containing pleckstrin (apparent M_r, 47 000) and the phosphorylated light chain of myosin (apparent M_r, 20 000) were cut out and counted for ^{32}P, which was measured as Cerenkov radiation in 0.01% (w/v) 4-methylumbelliferone. Incorporation of ^{32}P into both pleckstrin and myosin light chain was expressed as pmol/10^8 platelets.

Measurement of [^{3}H]inositol phosphate formation

In some experiments, platelets were labeled with both [^{3}H]inositol and [^{14}C]5-HT before permeabilization, to permit measurement of phosphoinositide-specific phospholipase C activity. Labeling with [^{3}H]inositol and measurement of the total formation of [^{3}H]inositol phosphates in permeabilized platelets were carried out essentially as described by Culty *et al.* (1988). However, the [^{3}H]inositol phosphates were eluted from Dowex-1 together, rather than individually, using 1.2 M ammonium formate in 0.1 M formic acid. LiCl was not included in these assays, because previous work showed that this salt decreased rather than increased the total recovery of [^{3}H]inositol phosphates in this system (Culty *et al.*, 1988).

Statistics

Incubations for measurement of secretion (or [^{3}H]inositol phosphate formation) were performed in triplicate and incubations for measurement of protein phosphorylation in duplicate; values from individual experiments are given as mean ± SE or as mean ± range, respectively. Experiments were repeated three or more times and pooled results are given as mean ± SE; the significance of differences was then determined by two-sided paired *t* tests.

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PAPER 2

Effects of inhibitors of protein kinase C on dense and α-granule secretion from electropermeabilized human platelets; inhibition of protein phosphorylation does not block secretion induced by Ca^{2+} with GTP[S]

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Abstract

5-Hydroxy[^{14}C]tryptamine (5-HT^{1})-labelled electropermeabilized human platelets suspended in a glutamate-based medium containing ATP were stimulated with 100 nM phorbol 12-myristate 13-acetate (PMA) and/or 100 μM guanosine 5'-O-(3-thiotriphosphate) (GTP[S]), in both the presence and absence of Ca^{2+} (pCa >9 - 4.5). [^{14}C]5-HT and β-thromboglobulin (βTG) release were used to measure the secretion of dense and α-granule constituents, respectively, and the phosphorylation of pleckstrin in permeabilized platelets incubated with [γ-^{32}P]ATP was used as an index of protein kinase (PKC) activity during secretion. Ca^{2+}-independent secretion of 5-HT in response to PMA and secretion of both 5-HT and βTG in response to GTP[S] were associated with the activation of PKC. These effects were blocked by 0.2 - 1 μM staurosporine, indicating that PKC-mediated protein phosphorylation was essential but not sufficient for secretion induced by GTP[S]. Complete inhibition of PKC activity by 4 μM staurosporine in the presence of high Ca^{2+} (pCa 4.5) abolished secretion of both 5-HT and βTG when Ca^{2+} alone or Ca^{2+} and PMA were present, but was largely ineffective in the presence of GTP[S]. These results show that GTP[S] can induce Ca^{2+}-dependent secretion in the complete absence of PKC activity, though some association between myosin light chain phosphorylation and secretion was found. This pattern of function and phosphorylation was confirmed using the PKC pseudosubstrate as an inhibitor. We have also shown that [^{3}H]diacylglycerol (DAG) formation in platelets labelled with

[^{3}H]arachidonate was stimulated by GTP[S] in the presence but not the absence of Ca^{2+}, indicating that Ca^{2+}-independent secretion and activation of PKC by GTP[S] may not depend on DAG formation. In the presence of intermediate Ca^{2+} concentrations (pCa 6), staurosporine enhanced DAG formation, while inhibiting PKC activity. Under these conditions, no secretion was observed in the presence of high DAG concentrations, indicating that DAG does not have a direct fusogenic effect.

Abbreviations: DAG, 1,2-diacylglycerol; GTP[S], guanosine 5'-O-(3-thiotriphosphate); 5-HT, 5-hydroxytryptamine; pCa, $-\log[Ca^{2+}_{free}]$; MLC, myosin light chain; PKC, protein kinase C; PKC pseudosubstrate, H_2N-Arg^{19}-Phe-Ala-Arg-Lys-Gly-Ala-Leu-Arg-Gln-Lys-Asn-Val-His- Glu-Val-Lys-Asn^{36}-OH; PMA, phorbol 12-myristate 13-acetate; βTG, β-thromboglobulin.

Introduction

Platelets contain three types of secretory granules that are distinct in both content and sensitivity to agonist-induced release [1-3]. Studies on electropermeabilized human platelets have provided detailed information concerning factors involved in the regulation of secretion from both dense granules and lysosomes [2,4,5]. A previous report from this laboratory [3] presented a detailed comparison of the factors affecting the secretion of both dense and α-granule constituents. This study showed that, even in the effective absence of Ca^{2+}, both guanosine 5'-O-(3-thiotriphosphate) (GTP[S]) and phorbol 12-myristate 13-acetate (PMA) could each produce a limited degree of secretion and that together these compounds acted synergistically, resulting in a maximal secretory response. The Ca^{2+}-independent effects of PMA and GTP[S] are associated with the activation of protein kinase C (PKC) as defined by the phosphorylation of pleckstrin (P47), the major substrate of PKC in platelets [6]. GTP[S] proved to have stimulatory effects on secretion from both granule types although these effects were somewhat more selective in the promotion of α-granule as compared to dense granule release [3]. Together with a report on secretion from permeabilized mast cells [7] these findings suggest that similar fundamental secretory mechanisms may mediate exocytosis in a variety of cell types. These mechanisms are likely to involve a putative GTP-binding protein, G_E [8], distinct from that involved in the activation of phosphoinositide-specific

phospholipase C (PLC).

In an effort to identify any PKC-independent effects of GTP[S], and characterize the relationship between protein phosphorylation and secretion, the potent but somewhat non-specific PKC inhibitor staurosporine [9,10] has now been employed. This compound appears to inhibit PKC by competing with ATP binding at the catalytic site [11] and has been used quite successfully in a variety of cell types [12-14] including platelets [15,16]. In thrombin-stimulated intact platelets staurosporine was found to inhibit shape change, secretion, and the phosphorylation of both pleckstrin and the myosin light chain (MLC) completely but only to slow aggregation [16]. For confirmation, some of the studies reported here were repeated using the PKC pseudosubstrate as an inhibitor, in order to rule out any possible non-specific effects of staurosporine. This peptide, analogous to the endogenous auto-inhibitory region of PKC-α has proven to be a potent and specific inhibitor of PKC activity both in vitro [17] and in permeabilized cells [14,18]. The results provide evidence that the interacting effects of three factors (Ca^{2+}, PKC and a G-protein-activated mechanism) mediate secretion from electropermeabilized platelets; any two factors could induce maximal secretion but no specific factor was essential under all conditions.

Results

Secretion and protein phosphorylation

As described previously [3], we have used [^{14}C]5-HT and βTG as markers for the secretion of platelet dense and α-granule constituents, respectively, and the phosphorylation of pleckstrin as an index of PKC activity during secretion. Figure 1A illustrates the Ca^{2+}-dependence of [^{14}C]5-HT secretion from permeabilized platelets incubated for 10 min at 25°C and the substantial increases in the Ca^{2+}-sensitivity of this process in the presence of PMA (100 nM) or GTP[S] (100 μM). Notably, PMA and GTP[S] are each capable of eliciting some degree of Ca^{2+}-independent (pCa >9) secretion from dense granules and these effects are potentiated synergistically when both compounds are added simultaneously.

Addition of Ca^{2+} ions alone to permeabilized platelets increased the phosphorylation of pleckstrin (Figures 1B, 2) [3,4] suggesting a Ca^{2+}-dependent activation of PKC, but this reaction did not correlate well with secretion. Similarly to their effects on secretion, PMA and GTP[S] individually promoted the Ca^{2+}-independent phosphorylation of pleckstrin and potentiated the phosphorylation observed in the presence of physiological concentrations of Ca^{2+} (pCa 7 - 6; Figures 1B, 2). In contrast, the addition of GTP[S] did not further increase the phosphorylation of pleckstrin caused by PMA, which was maximal at pCa >9 (Figure 1B). Thus, the supra-additive secretory effects produced by GTP[S] in combination with an optimal concentration of PMA (Figure 1A) [3], in comparison to those effects

seen on pleckstrin phosphorylation (Figure 1B), suggest that GTP[S] must act through some mechanism(s) in addition to the activation of PKC. It is evident that PMA and GTP[S] can each promote the phosphorylation of proteins in addition to pleckstrin (Figure 2), most notably the MLC (P20), although the dominant factor regulating the phosphorylation of this protein was Ca^{2+}. In the absence of Ca^{2+} (pCa >9) PMA was more effective than GTP[S] in stimulating protein phosphorylation, particularly of the polypeptides designated P39-41 [19]. In contrast, neither PMA or GTP[S] had any notable effect on the phosphorylation of either pleckstrin or the MLC at pCa 4.5 (Figures 1B, 2), a Ca^{2+} concentration previously shown to promote the optimum secretion of both [^{14}C]5-HT and βTG from permeabilized platelets [3].

Effects of staurosporine on PMA-induced secretion and phosphorylation

With the aim of isolating any PKC-independent effect(s) of GTP[S], and to examine the relationship between protein phosphorylation and secretion in more detail, we sought to inhibit phosphorylation, particularly that mediated by PKC. In both the absence and presence of Ca^{2+}, with or without PMA or GTP[S], staurosporine (4 μM) blocked the phosphorylation of both pleckstrin and the MLC; the increased labelling of other proteins was also suppressed (Figure 2).

In order to determine how the suppression of protein

phosphorylation might be related to function and whether the concentration of staurosporine might differentially influence these effects, permeabilized platelets were incubated in the absence or presence of Ca^{2+} and/or PMA and a range of staurosporine concentrations. In the absence of Ca^{2+}, complete inhibition of PMA-induced [^{14}C]5-HT secretion was seen with 0.2 μM staurosporine, whereas higher concentrations were required with Ca^{2+} alone or in the presence of PMA; in the latter cases, complete inhibition of secretion required 4 μM staurosporine (Figure 3A). No βTG secretion occurred at pCa >9 in the absence or presence of PMA (Figure 3B) [3]. βTG secretion at pCa 4.5, in the absence or presence of PMA, was inhibited in a manner parallel to that seen for inhibition of 5-HT secretion. It should be noted that 2 μM staurosporine appeared to be almost sufficient for the inhibition of both [^{14}C]5-HT and βTG secretion in the presence of Ca^{2+} alone, but that 4 μM staurosporine was required in the presence of Ca^{2+} with PMA.

Pleckstrin phosphorylation at pCa 4.5, both with and without PMA, was completely inhibited by 1 - 4 μM staurosporine (Figure 3C). These results show that secretion stimulated by Ca^{2+} or Ca^{2+} with PMA was less sensitive to inhibition by staurosporine than was pleckstrin phosphorylation. Concentrations of staurosporine that inhibited pleckstrin phosphorylation by more than 50% (e.g. 0.2 - 1 μM) caused limited or incomplete inhibition of Ca^{2+}-dependent secretion; only complete inhibition of pleckstrin phosphorylation was associated with inhibition of both [^{14}C]5-HT and βTG secretion.

In contrast, Ca^{2+}-independent pleckstrin phosphorylation produced by PMA was blocked only at the highest concentrations of staurosporine used, while [^{14}C]5-HT secretion was far more sensitive to the inhibitor (Figure 3). This suggests the possibility that, in the presence of Ca^{2+}, a low level of PKC activity is sufficient to support secretion whereas, in the absence of Ca^{2+}, a relatively high level of PKC activity is required.

In the absence of Ca^{2+}, PMA-induced phosphorylation of the MLC was completely suppressed by 0.2 - 1 μM staurosporine (Figure 3D) and Ca^{2+}-dependent phosphorylation, in both the absence and presence of PMA, was blocked by 4 μM staurosporine. This latter effect probably reflects inhibition of the MLC kinase in addition to PKC [15]. The results suggest some correlation between secretion and phosphorylation of the MLC in the presence of Ca^{2+}.

Effects of staurosporine on GTP[S]-induced secretion and phosphorylation

In contrast to the effects seen with PMA in the absence of Ca^{2+}, the complete inhibition of [^{14}C]5-HT secretion induced by GTP[S] alone required slightly higher levels of staurosporine (0.2 - 1 μM; Figure 4A). However, in the presence of Ca^{2+}, even 4 μM staurosporine blocked GTP[S]-induced secretion by only about 40% in 7 expts. (Figure 4A). The pattern of staurosporine-mediated inhibition of βTG secretion, in both the presence and absence of Ca^{2+}, closely paralleled that of [^{14}C]5-HT secretion (Figure 4B);

4 μM staurosporine blocked secretion in the presence of GTP[S] and Ca^{2+} by only about 30% in 6 expts. The extent of pleckstrin phosphorylation seen with GTP[S] alone (Figures 1B, 4C, 5C) was approximately half that seen with PMA alone (Figure 1B, 3C), whereas in the presence of Ca^{2+} the resulting phosphorylation differed to a lesser extent (Figures 1B, 4C, 5C). Pleckstrin phosphorylation induced by Ca^{2+}, GTP[S], and GTP[S] with Ca^{2+} was completely inhibited by 1 - 4 μM staurosporine (Figures 4C, 5C), an effect similar to that seen with PMA (Figure 3C). Thus, in the presence of Ca^{2+}, there was no correlation between secretion and pleckstrin phosphorylation, whereas in the absence of Ca^{2+} there was a good correlation. This indicates that PKC may be necessary for Ca^{2+}-independent secretion, but may have a more modulatory role under physiological conditions. Furthermore, a protein kinase-independent effect of GTP[S] appears to act in concert with Ca^{2+} to elicit substantial secretion from either dense or α-granules. Thus, for GTP[S] at pCa >9, the IC_{50} for secretion and pleckstrin phosphorylation was about 0.08 - 0.1 μM staurosporine. In contrast, at pCa 4.5, the IC_{50} for pleckstrin phosphorylation was similar to that mentioned above while secretion was inhibited by only 30 - 40%, even with 4 μM staurosporine. The GTP[S]-mediated phosphorylation of the MLC was inhibited by 0.2 - 1 μM staurosporine (Figure 4D) and, in the presence of Ca^{2+}, 1 - 4 μM of the inhibitor produced the same effect. Ca^{2+}-dependent phosphorylation of the MLC correlated fairly well with [^{14}C]5-HT but not βTG secretion, whereas

phosphorylation in the presence of GTP[S] and Ca^{2+} did not appear to correlate with the secretion of either marker.

Staurosporine (4 μM) completely suppressed the Ca^{2+}-dependent secretion of both [^{14}C]5-HT (Figure 5A) and βTG (Figure 5B) across a range of pCa values. However, at higher Ca^{2+} concentrations (pCa 6 - 4.5), staurosporine blocked the increased Ca^{2+}-sensitivity caused by 100 μM GTP[S], without suppressing secretion completely; addition of staurosporine with GTP[S] shifted the Ca^{2+} dose-response curve to the right (Figure 5A, B). This corresponded to an increase in the Ca^{2+} required for half-maximal secretion of both [^{14}C]5-HT and β-TG from pCa $\geq$ 7 for GTP[S] to pCa ~5.3 for GTP[S] with staurosporine, a value almost identical to that for half-maximal secretion caused by Ca^{2+} alone ([3] and this study). Consistent with previous findings that secretion from α-granules was more sensitive to GTP[S] than that from dense granules [3], the inhibition of βTG secretion required higher concentrations of staurosporine than did the inhibition of [^{14}C]5-HT secretion (Figure 4) and, with a maximal dose of staurosporine, GTP[S] promoted the secretion of βTG at pCa 6, whereas the inhibition of [^{14}C]5-HT secretion was not significantly overcome until pCa 5 (Figure 5).

GTP[S] also substantially increased the Ca^{2+}-sensitivity of pleckstrin phosphorylation across the pCa range studied; staurosporine completely suppressed pleckstrin phosphorylation under the same conditions (Figure 5C). Considering that the Ca^{2+}-independent effects of GTP[S] on both secretion and pleckstrin

phosphorylation were totally abolished by staurosporine, this again suggested an absolute requirement for PKC activity to facilitate secretion in the absence of Ca^{2+}. Staurosporine also inhibited phosphorylation of the MLC (Figure 5D), but there appeared to be no correlation between phosphorylation and secretion under these conditions.

In order to confirm the results obtained using staurosporine and to inhibit PKC more specifically, we compared the effects of staurosporine with those of a pseudosubstrate peptide corresponding to residues 19 - 36 of the type A PKC isozymes (Table 1). At pCa >9 the PKC pseudosubstrate potently inhibited both secretion and pleckstrin phosphorylation to an extent similar to that seen with 4 μM staurosporine. In the presence of Ca^{2+}, inhibition of both secretion and protein phosphorylation by the PKC pseudosubstrate was less complete than that seen with staurosporine. As well, inhibition of MLC phosphorylation was much less than that seen with staurosporine, probably reflecting the increased specificity of PKC inhibition by the pseudosubstrate. Thus, considering its inhibitory effects on both secretion and protein phosphorylation, which mimicked those seen with approximately 0.2 - 1 μM staurosporine (Figure 4; Table 1), the PKC pseudosubstrate confirmed all the data obtained with staurosporine, including the lack of correlation between secretion and pleckstrin phosphorylation in the presence of Ca^{2+}. Similar results were also obtained using a pseudosubstrate peptide five amino acids shorter, suggesting that penetration of the

permeabilized platelet was not a limiting factor (results not shown). Therefore, using two PKC inhibitors with quite different mechanisms of action, we have obtained evidence supporting the concept that Ca^{2+}, PKC and a G-protein-linked effector contribute to the control of secretion; no individual factor was essential to secretion under all conditions, but any combination of two was sufficient to support secretion.

We have also examined the effects of staurosporine on secretion and pleckstrin phosphorylation in platelets stimulated synergistically with PMA and GTP[S], in the absence of Ca^{2+} (Table 2). As shown (Figures 1, 3), PMA alone strongly stimulated pleckstrin phosphorylation and caused some secretion of [^{14}C]5-HT. In contrast, GTP[S] stimulated pleckstrin phosphorylation to only about 50% of that seen with PMA, but also caused limited secretion of both [^{14}C]5-HT and βTG (Figures 1, 4, 5). All these responses were completely suppressed by staurosporine (see also Figures 3, 4, 5). Together, PMA and GTP[S] stimulated pleckstrin phosphorylation to a level almost identical to that seen with PMA alone (Figure 1 and Table 2), and also promoted supra-additive increases in the secretion of both dense and α-granule constituents [3]. Staurosporine totally inhibited these synergistic responses (Table 2), again indicating a dependence on PKC for secretion in the absence of Ca^{2+}. These results were also confirmed using the shorter PKC pseudosubstrate peptide (results not shown).

Phospholipase C-mediated [^{3}H]DAG formation

We have previously shown that neither the Ca^{2+}-independent activation of PKC by GTP[S], nor the Ca^{2+}-independent secretion of 5-HT and βTG caused by GTP[S], in the presence or absence of PMA, are associated with PLC activity [3]. However, these findings did not exclude a role for DAG which, in addition to activating PKC, might potentiate secretion by alternative mechanisms [20,21]. We have now measured the PLC-mediated formation of [^{3}H]DAG in permeabilized platelets labelled with [^{3}H]arachidonic acid and incubated with Ca^{2+} concentrations known to cause minimal (pCa >9) [3] as well as optimal (pCa 6) [22] inositol phosphate formation (Table 3). In the absence of Ca^{2+}, there was no significant formation of DAG under any of the conditions tested and thus no correlation with the Ca^{2+}-independent secretion of [^{14}C]5-HT elicited by GTP[S], PMA or GTP[S] with PMA. GTP[S] alone, at pCa 6, induced significant [^{14}C]5-HT secretion but with limited [^{3}H]DAG formation; this formation of [^{3}H]DAG was stimulated by staurosporine whereas the secretion of [^{14}C]5-HT was strongly suppressed. PMA also enhanced the secretion of [^{14}C]5-HT at pCa 6, in the absence or presence of GTP[S], but partially inhibited [^{3}H]DAG formation with or without staurosporine. Whereas staurosporine suppressed the secretion of [^{14}C]5-HT induced by PMA with GTP[S] to below control levels (i.e., pCa 6 with no additions), the formation of [^{3}H]DAG under these conditions was four times higher than control. Although these results do not eliminate a possible role for DAG derived by

means other than the phosphoinositide-specific PLC, they do indicate that DAG formation is, in itself, an insufficient stimulus for secretion, as suggested by previous findings [23].

Discussion

In our studies of exocytotic mechanisms, we have now used the PKC inhibitor staurosporine to define better the factors mediating the secretion of dense and α-granule contituents from electropermeabilized human platelets. In the present study, staurosporine proved to be a potent inhibitor of PKC, as defined by the suppression of pleckstrin phosphorylation, at concentrations similar to those already reported [10,15,16].

At the highest concentrations used (2 - 4 μM), staurosporine not only completely suppressed pleckstrin phosphorylation but also inhibited that of the MLC (Figures 2 - 5), probably due in part to the inhibition of PKC, as well as to some cross-over inhibition of the MLC kinase [15]. In addition, staurosporine has been reported to inhibit other protein kinases including cAMP-dependent protein kinase and tyrosine kinases [10,24]. However, none of these protein kinase activities are likely to be involved in secretion at pCa >9, and recent evidence also argues against a primary role for protein tyrosine phosphorylation in platelet secretion [25]. Indeed, in our hands, the tyrosine kinase inhibitor genistein had no effect on secretion induced by GTP[S] or PMA in either the absence or presence of Ca^{2+} (Coorssen and Haslam, unpublished observations). In the

present study, staurosporine was used over a broad range of concentrations, the effects of which have previously been documented [10,15,16]. Furthermore, any nonspecific effects of staurosporine involving cytoskeletal disruption [26] might be expected to promote secretion; no stimulatory effects of staurosporine on secretion or protein phosphorylation [27] were observed in this study.

In the presence of PMA alone, PKC activation was maximal (Figure 3C), the MLC was only slightly phosphorylated (Figure 3D) and secretion (particularly that of βTG) is known to be quite slow [3]. The presence of Ca^{2+} results in substantial phosphorylation of the MLC which, in intact platelets, may play a role in granule centralization and thereby enhance granule fusion and release [28]. In addition, Ca^{2+} and activators of PKC are known to act synergistically to elicit a more rapid and complete secretory response [3,5,29]. Thus, the synergistic interaction of these two factors, Ca^{2+} and PKC, in the regulation of secretion has previously been characterized.

Ca^{2+} alone induced the secretion of both dense and α-granule constituents from permeabilized platelets, whereas the addition of GTP[S] or PMA shifted the Ca^{2+} dose-response curve to the left and caused small increases in the maximum secretion observed ([3,4] and this study). The high concentrations of PMA and GTP[S] used in these studies, in both the presence or absence of Ca^{2+}, were not meant to mimic any physiological conditions but rather were utilized as potent and selective agonists to analyze the influences of PKC

and GTP-binding proteins on secretion; restrictive conditions and maximal stimulation were used as means to dissect the secretory mechanism(s). Thus, in the absence of Ca^{2+}, PMA (100 nM) was found to stimulate a limited secretion of 5-HT, and GTP[S] (100 μM) to stimulate the secretion of both 5-HT and βTG (Figures 3 - 5; Tables 2, 3) [3]. Simultaneous addition of GTP[S] and PMA markedly enhanced these effects (Figure 1 and Tables 2, 3) [3]. PMA produced maximal phosphorylation of pleckstrin in the absence of Ca^{2+}, and GTP[S] was about half as effective (Figure 1C). Neither GTP[S] nor Ca^{2+} enhanced the phosphorylation of pleckstrin caused by PMA, and thus phosphorylation of this protein did not correlate well with secretion in this study or others [3,4]. Similar but much less pronounced effects were seen with the phosphorylation of the MLC which appeared to be associated with Ca^{2+}-dependent secretion in some cases (e.g. Figure 3). These studies therefore indicated that PKC activation promoted Ca^{2+}-independent secretion and that GTP[S], in the presence of Ca^{2+}, exerted additional effects on the secretion of both dense and α-granule constituents. These results are consistent with previous reports of GTP[S]-induced secretion from a variety of cells, independent of Ca^{2+}, polyphosphoinositide turnover, or PKC activity [3,7,30-33]. Gomperts and colleagues [8] have suggested that these effects are mediated by a distinct GTP-binding protein, G_E, that mediates a key late event in exocytosis. The possible presence of such a putative G protein in platelets was further explored in the present study.

The actions of GTP[S], particularly on the secretion of α-granule constituents, cannot be more than partly accounted for by the activation of PKC. In the absence of Ca^{2+} ions, GTP[S]-mediated secretion is associated with the phosphorylation of pleckstrin, but only to about half the extent seen with PMA (Figure 1B). Immunological evidence indicates that human platelets contain the α, β, δ and ζ isoforms of PKC [34-36]. PMA has been reported to function as a broad-spectrum activator of PKC isozymes in vitro, capable of stimulating substantial activity of α, β and δ isozymes even in the absence of Ca^{2+} [37]. Since the Ca^{2+}-independent isozymes δ and ζ differ from α and β in their regulatory as opposed to catalytic regions, they would be expected to show a comparable susceptibility to inhibition by staurosporine; this has, in part, recently been verified [38]. However, considering the differences in the regulatory domains, and the poor use by PKC-δ of a substrate peptide based on the PKC-α pseudosubstrate site compared to one based on its own unique pseudosubstrate site [39], it is likely that PKC-δ, and probably ζ, are not substantially inhibited by the pseudosubstrate peptides used in the present study.

The use of staurosporine has now provided evidence indicating that interactions between the effects of Ca^{2+}, PKC and a G-protein-regulated effector mediate secretion. No individual factor could produce an optimal secretory response but any combination of two could support maximal secretion. It has also been suggested that these factors interact synergistically under

physiological conditions [16]. Indeed, the potencies of these interactions are particularly evident for combinations of Ca^{2+} with PMA (Figure 3) or with GTP[S] (Figure 4). In both cases secretion was somewhat less sensitive to inhibition by staurosporine than was pleckstrin phosphorylation. This can be explained by one or more of three possible mechanisms. First, in light of the ability of staurosporine to shift the Ca^{2+}-sensitivity of GTP[S]-induced secretion back to the right (Figure 5), a more modulatory rather than essential role for PKC in secretion is suggested, probably involving the sensitization of the secretory mechanism to Ca^{2+}. Second, another kinase activity, less sensitive to staurosporine, may also play a role in secretion. Third, PKC may promote secretion by a mechanism independent of phosphorylation [40]. Activation of an effector enzyme by this mechanism may be influenced by staurosporine, which promotes the membrane translocation of PKC [41], but not by the pseudosubstrate peptide.

In the absence of Ca^{2+}, the PKC pseudosubstrate peptides inhibited both secretion and pleckstrin phosphorylation similarly to staurosporine, but were not as potent in the presence of Ca^{2+} (Figure 4; Table 1). We believe that this difference may in part be due to the 0°C equilibration used in our protocol, which is likely to limit the effective concentration of this peptide within the platelet. Indeed, the maximal entry of a similar peptide into T lymphocytes permeabilized with streptolysin-O (pores of macromolecular dimension compared to the ~2 nM diameter lesions produced by

electropermeabilization) required about 5 min [18]. Electropermeabilization in the presence of the pseudosubstrate probably results in greater uptake of the peptide due to the osmotic swelling characteristic of platelets in these preparations [4]. In addition, with strong activation at pCa 4.5 (Figure 1; Table 1), PKC may be less susceptible to inhibition by the pseudosubstrate peptides but still sensitive to high concentrations of staurosporine. Under these conditions, the γ and ζ PKC subspecies may also be activated (perhaps by the α and β species); therefore, the more potent inhibitions obtained with staurosporine may be due to its ability to inhibit all the isozymes of PKC present in the platelet. Furthermore, inhibition of PKC by the pseudosubstrate peptides at pCa >9 suggests the activation of α and β PKC isozymes under these Ca^{2+}-independent conditions, implying the production of a specific activator (see below).

The present results show that PKC is essential for the Ca^{2+}-independent secretion of both 5-HT and βTG seen with GTP[S]. These findings indicate that, even in the absence of Ca^{2+}, GTP[S] acts on a G-protein-mediated effector enzyme that produces a lipid second messenger capable of stimulating PKC. Alternatively, GTP[S] might stimulate Ca^{2+}-independent secretion through two different GTP-binding proteins, one capable of maintaining PKC activity and the other mediating a more direct control on secretion. However, since GTP[S] also has a fundamental role in the Ca^{2+}-dependent mechanism of secretion, independent of PKC, it is possible that the

product(s) of the effector enzyme(s) might stimulate secretion by an alternate mechanism, perhaps by promoting the contact of granule and plasma membranes and/or the site-specific perturbation and coalescence of these membranes. In this regard it is critical that, in conjunction with our previous study [3], we have now effectively eliminated both PLC as a potential effector enzyme and DAG as a fusogenic lipid in this system. The general lack of correlation between DAG levels and secretion (Table 3) appears to contradict the findings that accumulation of DAG is important for secondary aggregation and secretion [42]. Since secretion from electropermeabilized platelets is slow, and independent of aggregation, this may represent a genuine difference between intact and permeabilized platelets, although the authors of the latter study have also noted that the aggregatory response was more sensitive to exogenous DAG than was the secretory response. However, no effort was made to try and dissociate secretion from either aggregation or PKC activity.

Recent reports have shown that arachidonate and other fatty acids, particularly in combination with DAG, can activate PKC in both the presence and absence of Ca^{2+} [43,44]. The Ca^{2+}-independent activation of PKC by PA (with DAG) has also recently been characterized [38,45]. Therefore, possible effector enzymes that might generate PKC-activating and/or fusogenic lipids include phospholipase A_2 (PLA_2), phospholipase D (PLD) and a phosphatidylcholine-specific PLC (PC-PLC).

Considering the results presented here (Table 3) DAG is unlikely to be the mediator in question unless the species formed are distinct either biochemically, or in terms of subcellular location. Human platelets have also been shown to have very low PC-PLC activity [46]. Futhermore, despite substantial DAG formation, exogenous PLC did not stimulate secretion from permeabilized adrenal chromaffin cells [23]. Alternatively, human platelets are known to contain substantial agonist-induced PLA_2 activity which has recently been shown to parallel the secretion of βTG [47]. However, considering the evidence that there may be no essential difference between exocytosis from platelets and from mast cells [3,7] it is important to note that secretion has been dissociated from phospholipase A_2 activity in permeabilized mast cells [48]. It would appear that phospholipase D may be the most promising candidate for the role of effector in this system, and its immediate product, phosphatidate, has also proven to be potentially fusogenic [68,69]. A phosphorylation-independent mechanism of PLD activation by PKC has also been identified [40]. A role for phosphatidate or its metabolites in exocytosis from mast cells [50], pancreatic cells [51] and HL60 cells [52] has been proposed, and Bocckino et al. [53] have reported the stimulation of unidentified kinase activities by PA. Results from this laboratory [54] have shown that GTP[S], in both the absence and presence of Ca^{2+}, stimulates a phospholipase D activity in rabbit platelet membranes. PMA treatment potentiated the actions of GTP[S] on this PLD activity in a manner consistent

with the effect of PMA and GTP[S] on secretion from permeabilized human platelets. The presence of an agonist-stimulated PLD activity in human platelets has been established [55]. The significance of this PLD activity in relation to secretion of platelet granule constituents is currently under investigation, and studies have characterized strong correlations between this enzyme activity and secretion.

Materials and Methods

Materials

All materials and suppliers, with the exception of those listed below, were as previously noted in [3]. [^{3}H]Arachidonic acid was from DuPont Canada Inc. (Mississauga, Ont., Canada). Staurosporine was obtained from Boehringer Mannheim Canada Ltd. (Laval, Que. Canada). PKC pseudosubstrate was from Bachem Fine Chemicals, Inc. (Torrance, CA, U.S.A.) and Upstate Biotechnology Inc. (Lake Placid, NY, U.S.A.). Silica gel t.l.c. plates (Si250) were from the J.T. Baker Chemical Co. (Phillipsburg, NJ, U.S.A.)

Preparation of permeabilized human platelet suspensions

The isolation, [^{14}C]5-HT labelling, and electropermeabilization of human platelets was carried out as previously described [3,4]. Essentially, labelled platelets in Ca^{2+}-free Tyrode's solution (pH 6.5) containing 5 mM Pipes, 0.35% (w/v) bovine serum albumin and 5

mM EGTA were electropermeabilized in an apparatus set to deliver ten 3 kV discharges, and were then immediately cooled to 4°C. The permeabilized platelets were passed through a column of Sepharose CL-4B in a medium (pH 7.4) containing 12.5 mM $MgCl_2$ and the potassium salts of glutamic acid (160 mM), Hepes (20 mM), EGTA (2.5 mM) and EDTA (2.5 mM) (buffer A). Immediately following elution, the permeabilized platelet suspension was diluted with buffer A containing either unlabelled ATP or ATP + [γ-^{32}P]ATP (100 μCi/ml) to give 3 to 5 x 10^8 platelets/ml (final ATP, 5 mM) and stored at 0°C until used (within 2 h).

Incubations and measurment of secretion

As previously described [3], 160 μl aliquots of permeabilized platelet suspension were added to 40 μl of buffer A that had been adjusted to yield a specific final pCa value (at pH 7.4) and contained any required concentrations of GTP[S] and/or PKC pseudosubstrate. PMA, staurosporine or vehicle (0.4 μl of dimethylsulfoxide) were added simultaneously with the platelets. Mixtures were equilibrated for 15 min at 0°C and subsequently transferred to 25°C for 10 min. Incubations were terminated by addition of 200 μl of ice-cold buffer (pH 7.4) containing EDTA (35 mM), glutamate (80 mM) and Hepes (20 mM) and immediate centrifugation at 12,000 g for 1 min. Supernatants were quickly recovered for determination of [^{14}C]5-HT and βTG secretion, as described in [3]; [^{14}C]5-HT was measured by liquid scintillation and

βTG by radioimmunoassay. For experiments in which only the secretion of [^{14}C]5-HT was to be determined (Figure 1; Table 3), incubations were terminated by addition of 5 volumes of 0.15 M KCl containing 1.8% paraformaldehyde and 6 mM EDTA and subsequent centrifugation at 12,000 g for 1.5 min.

Phosphorylation of platelet proteins

Protein phosphorylation was measured using the methods of Haslam and Davidson [4]. Aliquots of permeabilized platelet suspension containing [γ-^{32}P]ATP were equilibrated and incubated with appropriate additions, as described above, and incubations were terminated by addition of 1.0 ml of 10% (w/v) trichloroacetic acid. Platelet protein was analyzed by SDS-PAGE and, following autoradiography, the regions of gel containing pleckstrin (apparent M_r, 47,000) and phosphorylated myosin light chain (apparent M_r, 20,000) were cut out and counted for ^{32}P, which was measured as Cerenkov radiation in 0.01% (w/v) 4-methylumbelliferone. As previously [3], incorporation of ^{32}P into both proteins was expressed as pmol/10^9 platelets.

Measurement of [^{3}H]diacylglycerol formation

According to the methods of Haslam and Davidson [4], platelets were labelled with both [^{14}C]5-HT and [^{3}H]arachidonic acid prior to permeabilization, in order to permit measurement of [^{3}H]DAG formation. Samples of appropriately labelled permeabilized platelet

suspension were equilibrated and incubated with appropriate additions, as described above. At the end of each incubation, 0.05 ml of suspension was taken for measurement of [^{14}C]5-HT secretion, as above. [^{3}H]DAG was extracted from the remaining suspension, purified by t.l.c. on silica gel (Si250) and counted for ^{3}H by liquid scintillation.

Statistics

Incubations for measurement of secretion (or [^{3}H]DAG formation) were performed in triplicate and incubations for measurement of protein phosphorylation in duplicate (unless otherwise noted); values from individual experiments are given as means ± S.E.M. or as means ± range, respectively. Experiments were repeated 3 or more times and pooled results are given as means ± S.E.M.; the significance of differences was then determined by two-sided paired t tests.

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Figure 1. Effects of PMA and GTP[S] on the secretion of [^{14}C]5-HT (A) from permeabilized platelets at various buffered Ca^{2+} concentrations; relationship to pleckstrin phosphorylation (B). Samples of permeabilized platelets in a glutamate-based medium containing ATP (see Materials and Methods) were equilibrated for 15 min at 0°C with the indicated Ca^{2+} buffers and either no other additions (○), 100 nM PMA (△), 100 μM GTP[S] (□) or 100 nM PMA and 100 μM GTP[S] (◇). The samples were then incubated for 10 min at 25°C. The secretion of [^{14}C]5-HT was measured in triplicate samples; mean values ± S.E.M. are shown. Single parallel incubations were conducted with permeabilized platelet suspension containing [γ-^{32}P]ATP for measurement of pleckstrin phosphorylation following SDS-PAGE. Areas of gels containing pleckstrin were cut out and counted for ^{32}P.

Figure 2. Effects of staurosporine on PMA and GTP[S]-stimulated protein phosphorylation in permeabilized platelets incubated in the absence and presence of Ca^{2+} ions. Samples of permeabilized platelet suspension containing [γ-^{32}P]ATP were equilibrated for 15 min at 0°C with 12.5 mM EGTA (pCa >9) or a Ca^{2+} buffer giving a pCa of 4.5 and either no stimulus, 100 nM PMA or 100 μM GTP[S]. In each case, the absence (-) or presence (+) of 4 μM staurosporine is noted. The samples were then incubated for 10 min at 25°C prior to the addition of trichloroacetic acid and analysis of ^{32}P-labelled proteins by SDS-PAGE. The first lane (far left) shows the protein phosphorylation pattern from a sample of unstimulated permeabilized platelet suspension that was neither equilibrated nor incubated.

Figure 3. Effects of increasing staurosporine concentrations on both control and PMA-stimulated secretion of [^{14}C]5-HT and βTG from permeabilized platelets incubated in the absence and presence of Ca^{2+} ions; relationship to both pleckstrin and myosin light chain phosphorylation. Samples of permeabilized platelet suspension containing either 12.5 mM EGTA (pCa >9; open symbols) or Ca^{2+} buffer giving a pCa of 4.5 (closed symbols) were equilibrated for 15 min at 0°C with the indicated concentrations of staurosporine in the absence (○, ●) or presence (△, ▲) of 100 nM PMA, and were then incubated for 10 min at 25°C. The secretion of [^{14}C]5-HT (A) and of βTG (B) were measured in three identical samples; mean values ± S.E.M. are shown. Parallel duplicate incubations were carried out with permeabilized platelet suspension containing [γ-^{32}P]ATP for measurement of the phosphorylation of pleckstrin (C) and myosin light chain (D); mean values ± range are shown.

Figure 4. Effects of increasing staurosporine concentrations on both control and GTP[S]-stimulated secretion of [^{14}C]5-HT and βTG from permeabilized platelets incubated in the absence and presence of Ca^{2+} ions; relationship to both pleckstrin and myosin light chain phosphorylation. Samples of permeabilized platelet suspension containing either 12.5 mM EGTA (pCa > 9; open symbols) or Ca^{2+} buffer giving a pCa of 4.5 (closed symbols) were equilibrated for 15 min at 0°C with the indicated concentrations of staurosporine in the absence (○, ●) or presence (□, ■) of 100 μM GTP[S], and were then incubated for 10 min at 25°C. The secretion of [^{14}C]5-HT (A) and of βTG (B) were measured in three identical samples; mean values ± S.E.M. are shown. Parallel duplicate incubations were carried out with permeabilized platelet suspension containing [γ-^{32}P]ATP for measurement of the phosphorylation of pleckstrin (C) and myosin light chain (D); mean values ± range are shown.

Figure 5. Effects of staurosporine and GTP[S] on the secretion of [^{14}C]5-HT and βTG from permeabilized platelets at various buffered Ca^{2+} concentrations; relationship to pleckstrin and myosin light chain phosphorylation. Samples of permeabilized platelet suspension were equilibrated for 15 min at 0°C with the indicated Ca^{2+} buffers and either no other additions (○), 4 μM staurosporine (●), 100 μM GTP[S] (□) or 100 μM GTP[S] with 4 μM staurosporine (■). The samples were then incubated for 10 min at 25°C. The secretion of [^{14}C]5-HT (A) and βTG (B) were measured in three identical samples; mean values ± S.E.M. are shown. Parallel duplicate incubations were carried out with permeabilized platelet suspension containing [γ-^{32}P]ATP for measurement of the phosphorylation of pleckstrin (C) and myosin light chain (D); mean values ± range are shown.

Fig. I

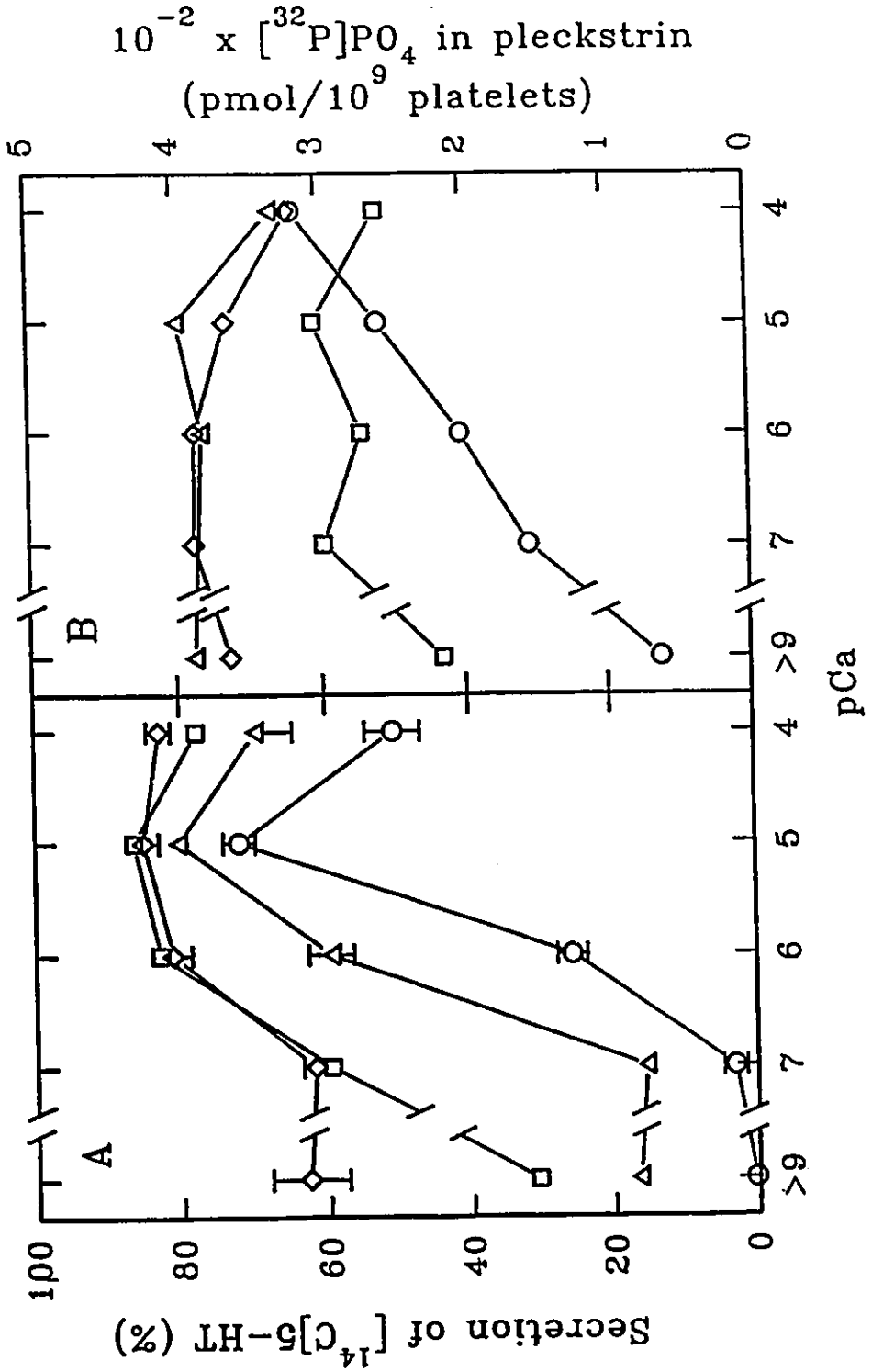
10^{-2} x [^{32}P]PO_4 in pleckstrin
(pmol/10^9 platelets)
5
4
3
2
1
0
B
A
4
5
6
7
>9
pCa
100
80
60
40
20
0
Secretion of [^{14}C]5-HT (%)

Fig. 2

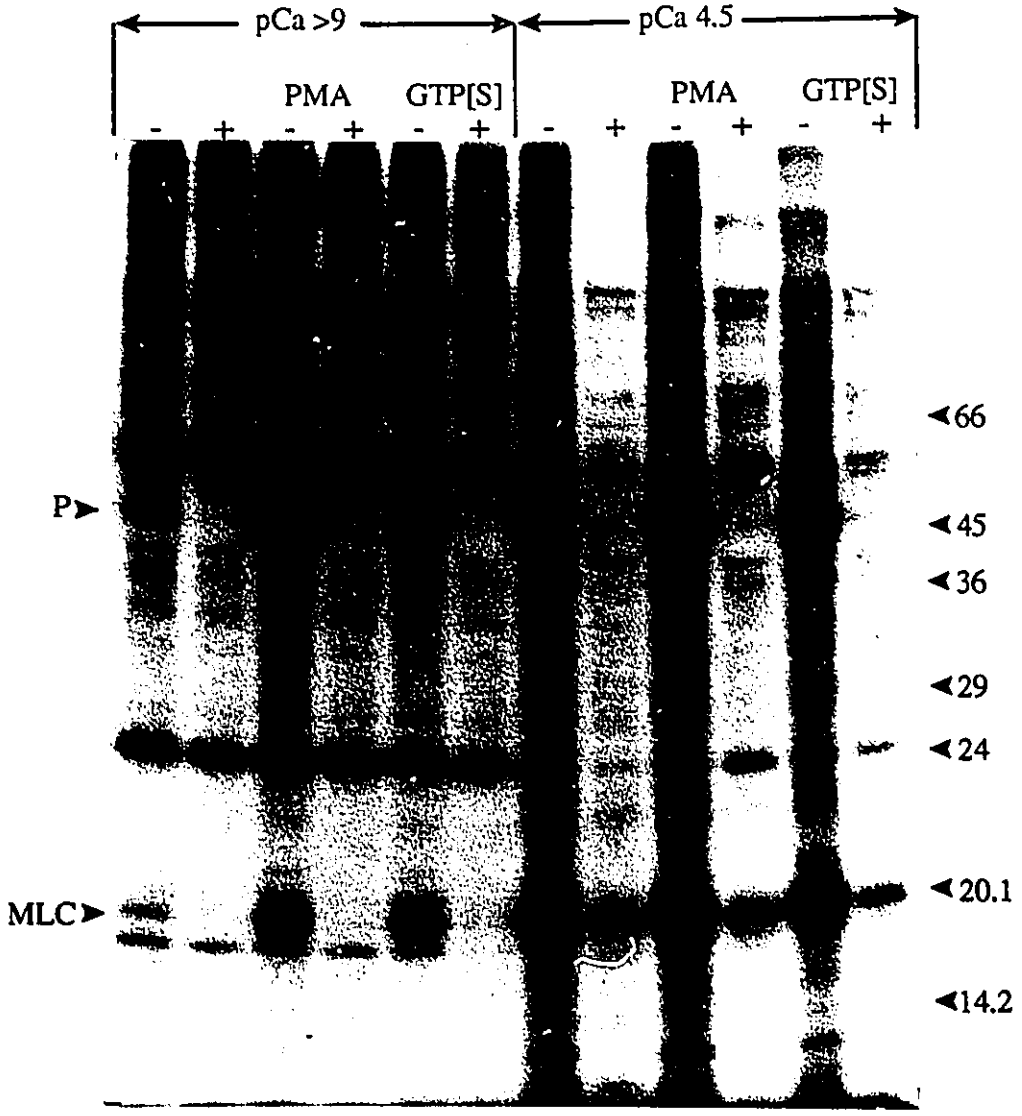

Fig. 3

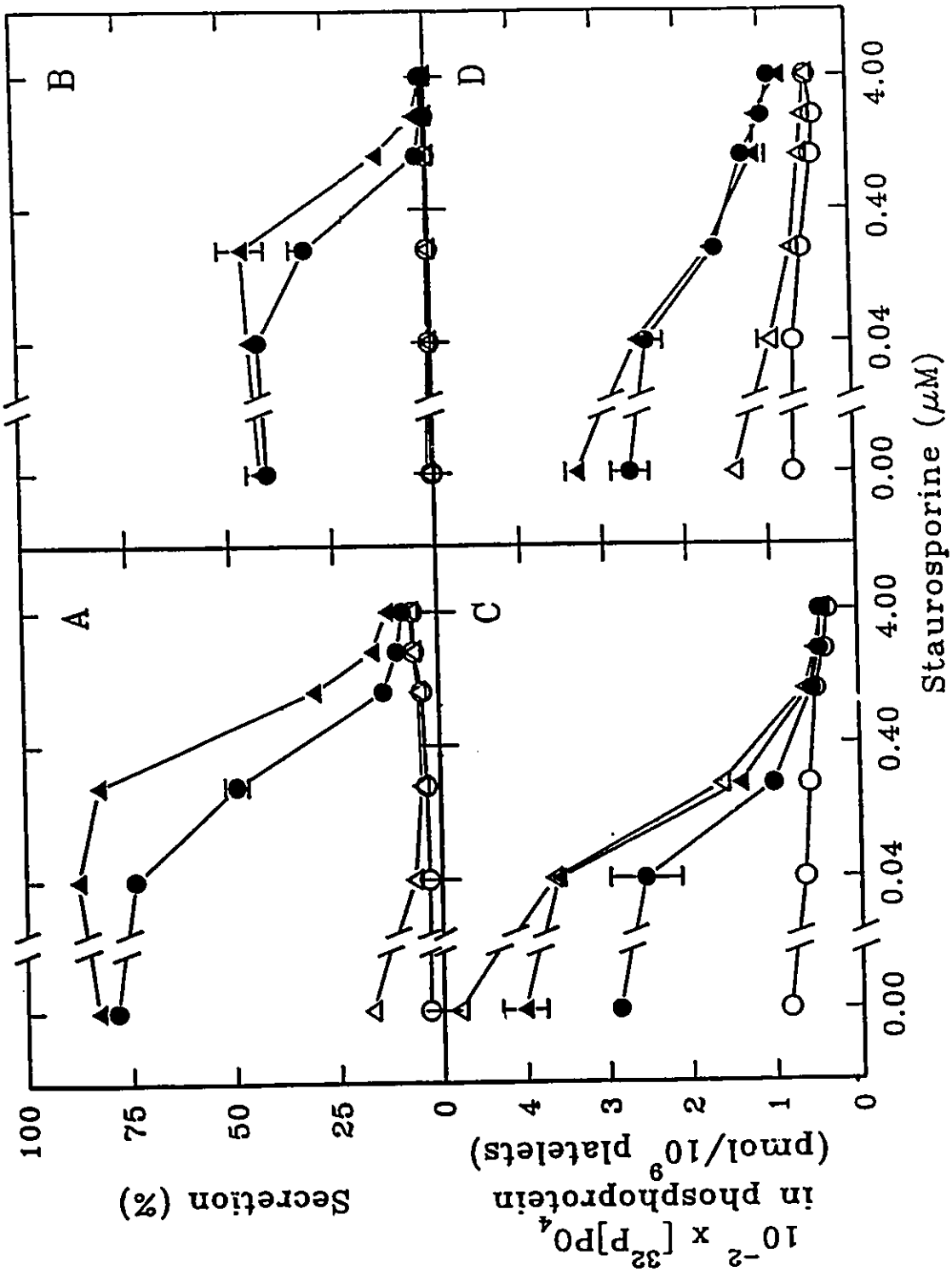
A
B
C
D
Secretion (%)
100
75
50
25
0
10^{-2} x [^{32}P]PO$_4$ in phosphoprotein (pmol/10^9 platelets)
4
3
2
1
0
0.00
0.04
0.40
4.00
Staurosporine (μM)

Fig. 4

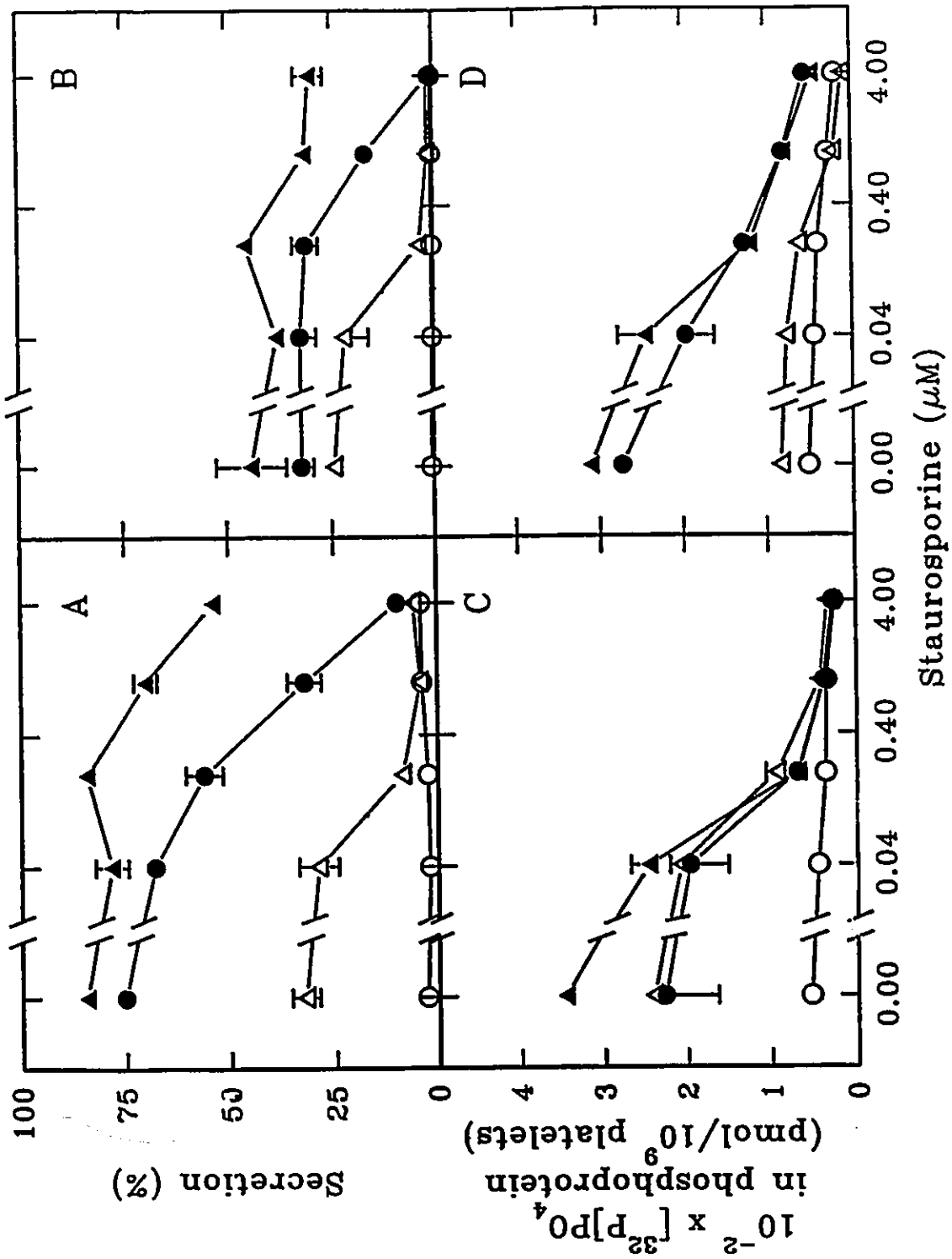
A
B
C
D
Secretion (%)
100
75
50
25
0
10^{-2} x [^{32}P]PO$_4$ in phosphoprotein (pmol/10^9 platelets)
4
3
2
1
0
0.00
0.04
0.40
4.00
Staurosporine (μM)

Fig. 5

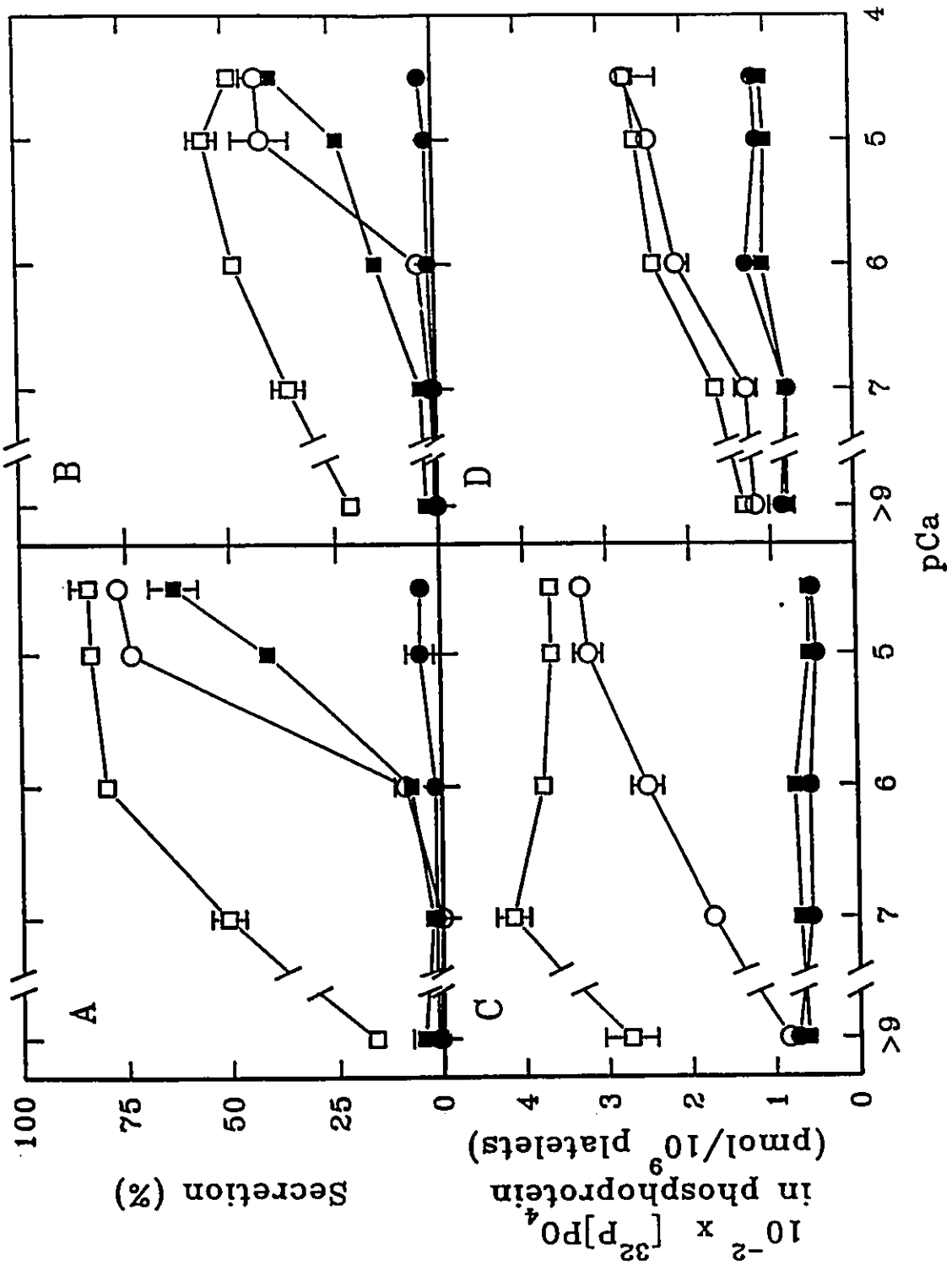
A
B
C
D
Secretion (%)
100
75
50
25
0
10^{-2} x [^{32}P]PO$_4$ in phosphoprotein (pmol/10^9 platelets)
4
3
2
1
0
4
5
6
7
>9
pCa

Table 1. Comparison of the effects of staurosporine and PKC pseudosubstrate on secretion and protein phosphorylation in permeabilized platelets

pCa	Additions	Secretion (%)		$[^{32}P]PO_4$ incorporated into protein (pmol/10^9 platelets)	
		$[^{14}C]$5-HT	βTG	Pleckstrin	Myosin light chain
>9	None	3 ± 0	0 ± 0	49 ± 1	45 ± 0
	Staurosporine	6 ± 0	0 ± 0	29 ± 0	31 ± 0
	Pseudosubstrate	5 ± 0	0 ± 0	35 ± 3	41 ± 1
	GTPγS	26 ± 0	23 ± 1	151 ± 11	67 ± 5
	GTPγS + staurosporine	7 ± 1	1 ± 0	25 ± 0	16 ± 2
	GTPγS + pseudosubstrate	8 ± 0	2 ± 0	59 ± 5	54 ± 1
4.5	None	72 ± 2	42 ± 6	216 ± 23	197 ± 4
	Staurosporine	10 ± 2	2 ± 0	25 ± 2	47 ± 5
	Pseudosubstrate	34 ± 1	16 ± 4	71 ± 1	122 ± 2
	GTPγS	90 ± 1	45 ± 2	253 ± 2	211 ± 13
	GTPγS + staurosporine	48 ± 1	20 ± 2	21 ± 3	31 ± 0
	GTPγS + pseudosubstrate	80 ± 1	36 ± 4	78 ± 6	118 ± 1

Samples of permeabilized platelet suspension, some containing [γ-^{32}P]ATP, were equilibrated for 15 min at 0°C with 12.5 mM EGTA (pCa >9) or with a Ca^{2+} buffer giving a pCa of 4.5, in each case in the presence of the above additions. The concentrations of these compounds were: staurosporine, 4 μM; PKC pseudosubstrate, 100 μM; GTPγS, 100 μM. The samples were then incubated for 10 min at 25°C. Secretion of $[^{14}C]$5-HT and βTG were measured in triplicate samples from which labelled ATP was omitted; values are means ± S.E.M. The incorporation of ^{32}P into pleckstrin and myosin light chain were determined in duplicate samples containing [γ-^{32}P]ATP; values are means ± range.

Table 2. Effects of staurosporine on the synergistic stimulation of secretion by PMA and GTPγS in the absence of Ca^{2+} ions

Additions	Secretion (%)		$[^{32}P]PO_4$ in pleckstrin (pmol/10^9 platelets)
	$[^{14}C]$5-HT	βTG	
None	0 ± 0	1 ± 0	57
Staurosporine	3 ± 1	1 ± 0	33
PMA	17 ± 0	3 ± 0	418
PMA + staurosporine	3 ± 0	1 ± 0	33
GTPγS	20 ± 1	14 ± 1	204
GTPγS + staurosporine	3 ± 0	3 ± 0	30
PMA + GTPγS	71 ± 1	46 ± 4	409
PMA + GTPγS + staurosporine	3 ± 0	2 ± 0	30

Samples of permeabilized platelet suspension containing 12.5 mM EGTA (pCa >9), and in some instances [γ-^{32}P]ATP, were equilibrated for 15 min at 0°C with the above additions. The concentrations of these compounds were; staurosporine, 4 μM; PMA, 100 nM; GTPγS, 100 μM. The samples were then incubated for 10 min at 25°C. Secretion of $[^{14}C]$5-HT and βTG were measured in triplicate samples from which labelled ATP had been omitted; values are means ± S.E.M. The incorporation of ^{32}P into pleckstrin was measured in identical samples equilibrated with [γ-^{32}P]ATP.

Table 3. Effects of Ca^{2+}, staurosporine and PMA on GTPγS-induced [^{14}C]5-HT secretion and [^{3}H]DAG formation in permeabilized platelets

pCa	Other Additions	Secretion of [^{14}C]5-HT (%)		10^{-3} x [^{3}H]DAG (d.p.m./10^{9} platelets)	
		- GTPγS	+ GTPγS	- GTPγS	+ GTPγS
>9	None	0 ± 0	23 ± 1	1.3 ± 0.0	1.7 ± 0.2
	Staurosporine	3 ± 0	3 ± 0	1.4 ± 0.1	1.4 ± 0.0
	PMA	16 ± 0	65 ± 1	1.1 ± 0.1	1.3 ± 0.1
	PMA + staurosporine	3 ± 0	3 ± 1	1.0 ± 0.1	0.9 ± 0.1
6	None	9 ± 1	84 ± 2	2.8 ± 0.3	14.1 ± 0.9
	Staurosporine	2 ± 0	5 ± 0	1.7 ± 0.1	21.4 ± 0.4
	PMA	50 ± 2	86 ± 0	1.3 ± 0.1	4.7 ± 0.1
	PMA + staurosporine	2 ± 0	4 ± 0	1.1 ± 0.1	11.6 ± 0.3

Platelets were labelled with both [^{14}C]5-HT and [^{3}H]arachidonate. After permeabilization and gel filtration of the platelets, samples of platelet suspension were equilibrated for 15 min at 0°C with 12.5 mM EGTA (pCa >9) or with Ca^{2+} buffer giving a pCa of 6, in each case in the presence of the additions listed above. The concentrations of these compounds were: staurosporine, 4 μM; PMA, 100 nM; GTPγS, 100 μM. The samples were then incubated for 10 min at 25°C and the secretion of [^{14}C]5-HT and the amount of [^{3}H]DAG present were determined. Results are means ± S.E.M. from triplicate samples.

PAPER 3

Correlations between the GTP[S]-induced activation of phospholipase D and dense granule secretion from permeabilized platelets

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ABSTRACT

In a previous study (Coorssen et al., 1990, *Cell Regulation 1*, 1027-1041), the combination of a nonhydrolysable GTP analogue (GTP[S])[1] and a phorbol ester (PMA) were shown to promote a marked secretory response in the effective absence of Ca^{2+} (pCa >9), a condition in which each compound alone had only moderate effects and phosphoinositide-specific phospholipase C (PI-PLC) was inactive. These results indicated that secretion from platelets was regulated by a GTP-binding protein (G_E) and a more recent study (Coorssen and Haslam, 1993, *FEBS Lett.*, in the press) showed that GTP[S] and PMA also had a synergistic effect on the activation of phospholipase D (PLD), suggesting that this enzyme might be the effector of G_E in platelets. In the present study, using electropermeabilized platelets containing [^{3}H]arachidonic acid-labelled phospholipids and dense granule [^{14}C]5-HT, no GTP[S]-induced accumulation of [^{3}H]diacylglycerol (DAG) was detected at pCa >9, although substantial accumulation occurred in the presence of Ca^{2+} (pCa 6). The latter [^{3}H]DAG formation was markedly inhibited by PMA and did not correlate with the secretion of dense granule [^{14}C]5-HT. GTP[S]-induced PLD activity, assayed by the formation of [^{3}H]PA and [^{3}H]PEt in the absence and presence of ethanol, respectively, correlated with secretion in both the presence and absence of Ca^{2+}. Ethanol inhibited both the maximal Ca^{2+}-independent PLD activity and secretion stimulated by GTP[S] with PMA in a similar concentration-dependent manner. The formation of [^{3}H]PEt

correlated with this inhibition and was always larger than the corresponding decrease in [^{3}H]PA formation. At pCa 6, the formation of [^{3}H]PA caused by GTP[S], PMA or a combination of these stimuli was also inhibited by increasing concentrations of ethanol, but only the secretion caused by PMA was inhibited. Increasing [^{3}H]PEt formation was seen with all stimuli in the presence of increasing concentrations of ethanol and [^{3}H]DAG formation was inhibited in parallel with the inhibition of [^{3}H]PA formation. These results suggested that PLD was the principal source of [^{3}H]PA in permeabilized platelets; this was confirmed using the potent DAG-kinase inhibitor, R59949. In order to analyze this apparent involvement of PLD in secretion, GTP[S] concentration-response studies were carried-out at pCa >9. In both the absence and presence of PMA, the formation of [^{3}H]PEt correlated with the secretion of [^{14}C]5-HT. Only [^{3}H]PA formed by PLD was associated with secretion. A time-course study at pCa >9 also showed that secretion induced by GTP[S] and/or PMA correlated with the formation of either [^{3}H]PA or [^{3}H]PEt, in the absence or presence of ethanol, respectively. PLD activation was not dependent on the presence of platelet secretory products in the supernatant. As a whole, the results of this study are consistent with our previous hypothesis that PLD may be the target of G_E in platelets and that PA produced by this enzyme exerts an important stimulatory effect on the exocytotic mechanism(s).

[1]Abbreviations: DAG, 1,2-diacylglycerol; GTP[S], guanosine 5'-o-(3-thiotriphosphate); 5-HT, 5-hydroxytryptamine; PA, phosphatidic acid; pCa, $-\log[Ca^{2+}_{free}]$; PEt, phosphatidylethanol; PKC, protein kinase C; PLC, phospholipase C; PLD, phospholipase D; PMA, phorbol 12-myristate 13-acetate; R59949, 3-(2-(4-(*bis*-(4-fluorophenyl)-methylene)-1-piperidinyl)-ethyl)-2,3-dihydro-2-thioxo-4(1H)-quinazolinone.

INTRODUCTION

Blood platelets possess three distinct secretory granule types that can be released in response to a variety of agonists [1]. Studies involving the use of electropermeabilized platelets to control internal divalent cation concentrations and to allow access of other small molecules including the nonhydrolysable GTP analogue, GTP[S], implicated Ca^{2+} ions [2] and the guanine nucleotide-dependent activation of phosphoinositide-specific phospholipase C (PI-PLC) and protein kinase C (PKC) [3,4,5] in the control of secretion from platelet dense granules. However, more recent studies designed to identify factors regulating the exocytosis of both dense and α-granule constituents [6], and of lysosomal enzymes [7], established that guanine nucleotides exert a more direct regulatory effect on the exocytotic mechanism(s) of platelets. Thus, using an effectively Ca^{2+}-free condition (pCa >9), GTP[S] was found to support limited secretion from both dense and α-granules, as well as maximal secretion when both GTP[S] and a phorbol ester (PMA) were added simultaneously [6]. The effects of GTP[S] could in part be explained by the activation of PKC, although PI-PLC activity was undetectable at pCa >9, suggesting that PKC was activated by a mechanism not involving DAG. As a whole, these studies have identified three factors (Ca^{2+}, PKC and a guanine nucleotide-regulated mechanism) that are involved in the control of secretion from human platelets. No single factor was either sufficient or essential under any experimental condition tested, but any

combination of two factors could produce a near maximal secretory response. In a variety of other permeabilized cell types including neutrophils [8], mast cells [9], chromaffin cells [10] and insulin-secreting RINm5F cells [11], similar data has collectively been interpreted as evidence for the existence of a distinct GTP-binding protein, G_E, closely coupled to the final stage(s) exocytosis [12,13]. Thus, the secretory mechanism(s) in platelets appears to be essentially similar to that of other cells in which a role for G_E has already been established.

With the role for a putative G_E protein in platelets confirmed, we sought to identify an effector enzyme regulated by this protein. It seemed likely that the product(s) of this effector could activate PKC, but might also have functions inherent to more terminal events in the exocytotic process, such as membrane fusion. Considering the dissociation of PI-PLC activity from secretion [6] and the finding that there is little PC-specific PLC activity in human platelets [14], it seemed unlikely that a PLC was the effector in question, although an alternative, fusogenic role for DAG in the secretory process could not be excluded [15,16]. In addition, all available evidence suggested that the cytosolic phospholipase A_2 associated with signal transduction in human platelets was Ca^{2+}-dependent [17,18]. However, a previous report from this laboratory [19] established that Ca^{2+}-independent phospholipase D (PLD) activity in rabbit platelet membranes was stimulated synergistically by GTP[S] and PMA in

a manner comparable to that seen for the stimulation of secretion from permeabilized human platelets, and it was suggested that activation of PLD might therefore be linked to secretion [6]. A role for PLD activation in secretion has also been suggested in mast cells [20], pancreatic cells [21] and HL60 cells [22]. The existence of a PLD activity in human platelets was established by Rubin [23] and quantitated by Huang et. al. [24].

We have recently shown that strong correlations exist between PLD activity and Ca^{2+}-independent secretion from permeabilized platelets [25]. This study established that both PLD activity and secretion are inhibited in a parallel, dose-dependent manner by BAPTA and analogues; this inhibition was unlikely to be due to the ability of BAPTA to bind Ca^{2+}, and may represent a direct effect of BAPTA on G_E or PLD. In the present study, we have further characterized the relationship between PLD activity and secretion to determine the extent to which the effects of the putative G_E can be explained by PLD as an effector enzyme. Under all circumstances, secretion was associated with PLD activity and the formation of phosphatidic acid (PA) and phosphatidylethanol (PEt) in the absence and presence of ethanol, respectively. Although PA may be formed and metabolized by several pathways, formation of the more stable metabolite PEt, by transphosphatidylation, is a definitive indicator of PLD activity [26].

EXPERIMENTAL

Materials

[5,6,8,9,11,12,14,15-^{3}H]Arachidonic acid (100 Ci/mmol) was obtained from Du Pont (Mississauga, Ont., Canada) and [*side-chain*-2-^{14}C]5-HT (55 mCi/mmol) from Amersham (Oakville, Ont., Canada). GTP[S] was supplied by Boehringer Mannheim (Laval, Que., Canada) and PMA by Sigma (St. Louis, MO, U.S.A.). Other materials were from sources listed in [3,6,19].

Labelling and electropermeabilization of platelets

Human platelets were isolated as previously described [3,6], resuspended at 5 x 10^{9}/ml in citrated plasma (pH 6.5) and incubated at 37°C for 1.5 h with [^{3}H]arachidonate (5 μCi/ml). During the last 30 min of labelling [^{14}C]5-HT was added in three equal amounts, to a final concentration of 0.25 μCi/ml. The platelets were then washed in Ca^{2+}-free Tyrode's solution containing 5 mM-Pipes (pH 6.5), 0.35% (w/v) BSA, 50 units of heparin/ml and apyrase (30 μM/ml). Following centrifugation, the platelets were resuspended in the same medium (without heparin or apyrase) and, after addition of 5 mM-EGTA, were electropermeabilized by high voltage electric discharges [3,6]. The permeabilized platelets were cooled to 4°C, freed from low-M_r solutes by gel filtration [3] and eluted in a medium (pH 7.4) containing 3.9 mM-$MgCl_2$ and the potassium salts of glutamic acid (160 mM), Hepes (20 mM), EDTA (2.5 mM) and EGTA (2.5 mM). The eluate was adjusted to

contain 5 x 10^8 platelets/ml following the addition of ATP (5 mM, final concentration). This suspension was stored at 0°C until used (30–60 min).

Incubations

All samples of permeabilized platelet suspension (1.6 x 10^8 platelets) were equilibrated for 15 min at 0°C with either an additional 10 mM–EGTA (pCa >9 even if all platelet Ca^{2+} were released [6]) or with a Ca^{2+} buffer (pCa 6); sufficient $MgCl_2$ was added to all samples to give 5 mM–Mg^{2+}_{free}, and any other additions were also made prior to equilibration. Concentrations of added $MgCl_2$ and $CaCl_2$ were calculated using binding constants given elsewhere [27]. After equilibration, samples (0.4 ml final vol.) were incubated at 25°C for 10 min, unless otherwise stated. Following incubation, a 0.05 ml sample of each incubation mixture was mixed with ice–cold formaldehyde–EDTA and the secretion of [^{14}C]5–HT measured by liquid scintillation counting [3]. Lipids were extracted from the remainder of each incubation mixture [28]. [^{3}H]PA and [^{3}H]PEt were isolated by t.l.c. [29], localized by iodine staining and counted for ^{3}H as previously described [19]. [^{3}H]DAG was measured using the same t.l.c. protocol as for [^{3}H]PA and [^{3}H]PEt, and these determinations correlated with those previously obtained using another t.l.c. system [30]. In order to characterize the labelling produced by the [^{3}H]arachidonate protocol, lipids were extracted from unincubated control samples and the phospholipid species

separated by t.l.c. in a solvent containing chloroform/methanol/40% methylamine (90:41:7.5, by vol.) [31] and counted for ^{3}H. Basal values for extracellular [^{14}C]5-HT and for ^{3}H-labelled lipids were obtained from permeabilized platelet suspension stored at 0°C and subtracted from values obtained after incubations. These corrections were very small for [^{14}C]5-HT and [^{3}H]PEt, whereas significant amounts of both [^{3}H]PA and [^{3}H]DAG were present prior to incubations. To normalize results from different experiments, ^{3}H-labelled lipids were expressed as percentages of total platelet ^{3}H.

Data presentation

In individual experiments, each incubation condition was in triplicate and each experiment was carried out at least two or three times. Values from representative experiments are given as means ± S.E. Pooled results from a number of experiments are given in the text as mean ± S.E.M. and the significance of differences were determined by two-sided paired *t* tests.

RESULTS

Labelling of platelet phospholipids

Comparisons of labelling intact human platelets with [^{3}H]glycerol, [^{3}H]myristate, [^{3}H]palmitate and [^{3}H]oleate under the conditions described in the Experimental section showed that [^{3}H]arachidonate provided the most rapid and extensive labelling, with low background for the determination of

phospholipase activities, although all labels could be used to measure PLD activity (data not shown). Expressed as percentages of total platelet ^{3}H, phosphatidylcholine was the most highly labelled phospholipid (42 ± 1%; mean ± S.E.), followed by phosphatidylethanolamine (14 ± 1%), phosphatidylserine (8 ± 0%) and phosphatidylinositol (7 ± 0%) in 26 experiments using the [^{3}H]arachidonate labelling protocol.

Secretion and the accumulation of DAG

As previously described [6], the incubation of electropermeabilized human platelets with GTP[S] (100 μM) or PMA (100 nM) resulted in limited Ca^{2+}-independent secretion of [^{14}C]5-HT (Tables 1,2), although together these compounds acted synergistically, resulting in a near maximal secretory response. Although Ca^{2+} alone (pCa 6) caused only about 10% secretion, GTP[S] in combination with Ca^{2+} caused a full secretory response and thus no synergistic interactions with PMA were observed; PMA alone caused about 50% secretion in the presence of Ca^{2+} (Tables 1,2). In contrast to their effects on secretion, GTP[S] and/or PMA did not cause any accumulation of [^{3}H]DAG at pCa >9 (Table 1). Although there was evidence of [^{3}H]DAG formation at pCa >9, there was no detectable accumulation in 6 out of 8 experiments using GTP[S] and/or PMA as stimuli. The accumulation of [^{3}H]DAG was maximal in response to stimulation by GTP[S], whereas addition of GTP[S] and PMA resulted in substantially lower

concentrations of [^{3}H]DAG (Table 1), presumably due to the inhibition of PI-PLC associated with the application of PMA [6]. Thus, [^{3}H]DAG accumulation did not correlate with the secretion of [^{14}C]5-HT in either the absence or presence of Ca^{2+}.

Correlations between secretion and PLD activity

The synergistic enhancement of [^{14}C]5-HT secretion caused by GTP[S] with PMA at pCa >9 was found to correlate with a similar increase in the concentrations of [^{3}H]PA and [^{3}H]PEt, in the absence and presence of ethanol (200 mM), respectively (Table 2). Only [^{3}H]PA formed in response to stimulation by GTP[S] and/or PMA was associated with the secretion of [^{14}C]5-HT. The limited secretion of [^{14}C]5-HT caused by Ca^{2+} alone (pCa 6) was also associated with the formation of [^{3}H]PA (Table 2). The synergistic combination of Ca^{2+} and GTP[S] produced, on average, a 2.5-fold increase in [^{3}H]PA accumulation (mean ± S.E.; n = 5) and a full secretory response. In the presence of Ca^{2+}, PMA caused 47 ± 3% secretion and [^{3}H]PA formation was only half (49 ± 4%) that observed with GTP[S] (means ± S.E.; n = 5). In five experiments, the combination of GTP[S] and PMA in the presence of Ca^{2+} did not enhance secretion or the formation of [^{3}H]PA beyond that seen with GTP[S] and Ca^{2+}. Thus, in both the absence and presence of Ca^{2+}, only [^{3}H]PA formed in response to stimulation by GTP[S] and/or PMA correlated with the secretion of [^{14}C]5-HT. Ethanol (200 mM) was used to analyze the

involvement of PLD in the formation of this PA associated with secretion. Ethanol caused only minor inhibition of Ca^{2+}-independent [^{14}C]5-HT secretion and [^{3}H]PA formation stimulated by GTP[S] and/or PMA (Table 2) [25]. In each case, the residual secretion correlated with the remaining [^{3}H]PA and with the corresponding formation of [^{3}H]PEt, which was always larger than the decrease in [^{3}H]PA caused by ethanol, suggesting that PLD was involved in the formation of [^{3}H]PA. In contrast, secretion stimulated by GTP[S] with Ca^{2+}, in the presence or absence of PMA, was unaffected by 200 mM-ethanol, whereas secretion induced by PMA was inhibited 17 ± 1% (mean ± S.E.; n = 5). However, in all cases, Ca^{2+}-dependent secretion correlated with the formation of [^{3}H]PEt in the presence of ethanol. Thus, only with PMA did the inhibition of [^{3}H]PA formation by ethanol in the presence of Ca^{2+} correlate with an inhibition of secretion. In order to further examine this relationship between secretion and PLD activity, ethanol dose-response studies were carried out in both the absence and presence of Ca^{2+}.

In the absence of Ca^{2+} and other additions, ethanol had little effect on secretion (Fig. 1a) or on the formation of either [^{3}H]PA or [^{3}H]PEt (Figs. 1b and c, respectively). In contrast, the strong secretory response produced by the synergistic combination of GTP[S] and PMA was inhibited in a concentration-dependent manner by ethanol and this was parallelled by a steady increase in [^{3}H]PEt formation which plateaued between 300 – 400 mM-ethanol. In parallel with this

inhibition of secretion, the formation of [^{3}H]PA in response to GTP[S] with PMA progressively decreased across the ethanol range used (Fig. 1b). The smaller effects produced by either GTP[S] or PMA showed similar patterns of inhibition by ethanol, but were omitted for clarity.

Secretion induced by PMA and Ca^{2+} was also progressively inhibited by increasing ethanol concentrations (Fig. 2a); 400 mM-ethanol caused about 40% inhibition. The amounts of [^{3}H]PA and [^{3}H]DAG were very similar to control values (Figs. 2b,d) in the presence of PMA, although a slight increase in [^{3}H]PEt was observed (Fig. 2c). As shown in Table 2, GTP[S] and GTP[S] with PMA had almost identical effects on both secretion and PLD activity in the presence of Ca^{2+} (Figs. 2a,b,c) and secretion was not inhibited by ethanol under these conditions. In contrast, progressive inhibition of [^{3}H]PA formation stimulated by GTP[S], in the absence or presence of PMA, resulted in inhibitions of 42 ± 10% and 53 ± 9% (means ± S.E.; n = 3), respectively, by 400 mM ethanol; [^{3}H]PEt levels increased in parallel by 98 ± 1% and 97 ± 2% (means ± S.E.M.; n = 3), indicating that much of the PA in permeabilized platelets was produced by PLD (Figs. 2b,c). The formation of [^{3}H]DAG was only observed in reponse to stimulation by GTP[S], whereas addition of GTP[S] and PMA resulted in substantially lower levels of [^{3}H]DAG, presumably due to the inhibition of PLC in the presence of PMA [6]. Thus, although [^{3}H]DAG formation in response to GTP[S] or GTP[S] and PMA was inhibited to a similar extent by increasing concentrations of

ethanol, only that formed in the presence of PMA was inhibited completely by 400 mM-ethanol. Since 400 mM-ethanol was sufficient to block the formation of [^{3}H]PA by PLD in this system (Figs. 2b,c), the [^{3}H]PA remaining was probably from another source, possibly from PLC (Fig. 2d) via the DAG-kinase pathway. Measurement of [^{32}P]PA formation in permeabilized platelets equilibrated with [γ-^{32}P]ATP confirmed this and also showed that 10 μM of the DAG-kinase inhibitor R59949 [32] only moderately inhibited the formation of [^{32}P]PA stimulated by GTP[S] at pCa 6, with no effect on secretion or [^{3}H]PA formation (data not shown). Both R59949 and propranolol (200 μM; a putative PA-phosphohydrolase inhibitor [33]) produced nonspecific effects; in addition to inhibiting PKC (measured as the phosphorylation of pleckstrin) in both the absence and presence of Ca^{2+}, propranolol also inhibited DAG-kinase more potently than did R59949 at pCa 6. Thus, although these inhibitors proved to be less selective than previously described [32,33], their effects suggested that most of the PA in permeabilized platelets was derived from phosphatidylcholine by PLD activity rather than from phosphatidylinositol by the sequential actions of PLC and DAG-kinase, and that DAG-kinase and PA-phosphohydrolase activities had little effect on secretion under conditions of optimal stimulation.

Dose-dependent effects of GTP[S] and PMA

Since there was no detectable PI-PLC activity in the

absence of Ca^{2+} (Fig. 2d) [6], dose-response studies for GTP[S] and PMA were carried out at pCa >9 to further analyze the relationship between PLD activity and secretion. GTP[S] caused small dose-dependent increases in both [^{14}C]5-HT secretion and PLD activity in the absence of Ca^{2+} (Fig. 3). Addition of 10 nM-PMA with increasing concentrations of GTP[S] enhanced both [^{14}C]5-HT secretion (Fig. 3a) and [^{3}H]PEt formation in the presence of 200 mM-ethanol (Fig. 3b), particularly between 3 - 100 μM-GTP[S]. With 100 nM-PMA, significant enhancement of both secretion and [^{3}H]PEt formation were observed with as little as 1 μM-GTP[S]. However, the difference between the effects of 10 nM and 100 nM PMA was much more pronounced with respect to the GTP[S]-induced activation of PLD than of secretion (Figs. 3a,b). For example, in the presence of 100 μM-GTP[S], 10 nM-PMA caused 39 ± 4% secretion and the conversion of 0.13 ± 0.02% of the total platelet ^{3}H to [^{3}H]PEt, whereas 100 nM-PMA caused corresponding changes of 47 ± 5% and 0.16 ± 0.03% (means ± S.E.M.; $n = 3$); the values for maximal secretion are not significantly different while those for [^{3}H]PEt formation are ($p < 0.05$). Corresponding effects of GTP[S] and PMA on both secretion and the formation of [^{3}H]PA were observed in the absence of ethanol.

The results from the experiment shown in Figs. 3a and b were replotted to examine the relationship between secretion and PLD activity, making secretion a function of [^{3}H]PEt and [^{3}H]PA accumulation in the presence or absence of ethanol,

respectively (Figs. 3c,d). This definitively shows that only [^{3}H]PA formed in response to the stimulation of PLD was associated with secretion (Fig. 3d), and that no PLD activity ([^{3}H]PEt formation) was observed under basal conditions (Fig. 3c). In the presence of 0, 10 or 100 nM-PMA, [^{14}C]5-HT secretion increased almost linearly as a function of the enhancement of PLD activity in the presence of increasing concentrations of GTP[S]. In the presence of 10 nM-PMA both the [^{3}H]PEt and [^{3}H]PA dose-responses of secretion were to the left of those observed in the presence of 100 nM-PMA, suggesting an inhibitory component to the action of the higher PMA concentration. However, the close correlations between PLD activity and secretion are consistent with a role for PA (and possibly of PEt) in secretion.

Time-course analysis

The relationship between PLD activity and secretion was further examined in a time-course (Fig. 4). This study was also conducted at pCa >9, since secretion under this condition was known to be slow enough [6] to permit a detailed analysis. Furthermore, since PA concentrations in incubated control samples were never associated with secretion (Fig. 3d), these PA values were subtracted from those obtained following stimulation with GTP[S], PMA or GTP[S] with PMA. GTP[S] alone induced a slow secretion of [^{14}C]5-HT that proceeded almost linearly for 20 min, apart from an initial 2-min lag (Fig. 4a) [6]. PMA alone produced little secretion, even after

incubation for 20 min (Fig. 4a). In contrast, no lag was seen after the simultaneous addition of both GTP[S] and PMA, which caused a much more rapid and extensive secretory response. The formation of [^{3}H]PA (Fig. 4b) correlated with the secretion of [^{14}C]5-HT throughout the time-course, under all three stimulus conditions. In the presence of 200 mM-ethanol the formation of [^{3}H]PEt was also associated with secretion induced by GTP[S] and/or PMA throughout the time-course, confirming that PLD activity was closely associated with the secretion of [^{14}C]5-HT. However, these results could also indicate that PLD activity is stimulated by secreted granule constituents or platelet products such as 5-HT or eicosanoids. Use of (i) the potent 5-HT receptor antagonist ritanserin (500 nM); (ii) the cyclooxygenase inhibitors indomethacin (10 μM) or ASA (200 μM); or (iii) the dual lipoxygenase/ cyclooxygenase inhibitor BW755C (100 μM) [34] had no effect on either Ca^{2+}-independent secretion or PLD activity (not shown), confirming that the stimulation of PLD was associated with secretion rather than a result of secretory products in the supernatant.

DISCUSSION

The present study establishes that strong correlations exist between PLD activity and secretion in both the absence and presence of Ca^{2+}, consistent with our previous hypothesis that PLD is the target of G_E in platelets [6,25,35]. The accumulation of [^{3}H]DAG did not correlate with secretion in either the absence or presence of Ca^{2+}, suggesting that DAG did not have any direct membrane-fusogenic effects [15,16] in this system. Although GTP[S] and PMA both had limited effects on their own, together these compounds acted synergistically to stimulate parallel Ca^{2+}-independent PLD activity and 5-HT secretion. In the presence of Ca^{2+}, GTP[S] alone produced a full secretory response and maximal activation of PLD; PMA was about half as effective as GTP[S] under these conditions. These responses are probably due to an enhancement of the secretory rate by Ca^{2+} [6] rather than to an essential effect of Ca^{2+} on the exocytotic mechanism, since (i) GTP[S] and PMA could act synergistically to stimulate both PLD activity and a slow but marked secretory response in the effective absence of Ca^{2+} (pCa >9); and (ii) Ca^{2+} at pCa 6 caused only limited secretion and activation of PLD.

In the absence of Ca^{2+}, ethanol caused parallel dose-dependent inhibitions of both secretion and PA formation stimulated by GTP[S] and/or PMA. The decrease in PA formation was attributable to the PLD-catalyzed formation of PEt by transphosphatidylation. Inhibition of the marked PA formation stimulated by GTP[S] with PMA was essentially complete at 400

mM-ethanol. Since higher ethanol concentrations caused slight increases in 5-HT secretion in the absence of other additions (Fig. 1a), the inhibition of GTP[S]-stimulated secretion is unlikely to represent a nonspecific effect of ethanol on the exocytotic mechanism(s). GTP[S] alone is known to induce effects that stimulate half-maximal PKC activity [6] that is essential for Ca^{2+}-independent secretion from permeabilized platelets [35]; the inhibition of secretion by ethanol in parallel with the formation of PEt indicates that PA is likely to be an activator of PKC and/or other kinases in the platelet [35,36]. Since the strong Ca^{2+}-independent secretory response produced by GTP[S] with PMA was only inhibited by about 50% at 400 mM-ethanol (Fig. 2) [25], and since DAG was not formed under these conditions, it appears that PEt may, to some degree, be capable of substituting for PA, perhaps by stimulating PKC [35,37]. Although PMA with Ca^{2+} had no effect on DAG accumulation (PLC activity), and little effect on PA formation, these agonists produced a substantial secretory response that was inhibited in a dose-dependent manner by ethanol and was associated with a moderate increase in PEt formation. Since the concentration of PMA used in these studies (100 nM) is known to cause as complete inhibition of PI-PLC activity [6], the majority of PA formed in the presence of PMA must be from PLD. Thus, in the presence of Ca^{2+}, the DAG formed by GTP[S] with PMA is substantially less than that formed by GTP[S] alone (Fig. 2c), presumably due to the absence of PLC activity [6]; ethanol caused a similar dose-

dependent inhibition of GTP[S]-induced DAG formation, in both the absence and presence of PMA, consistent with the inhibition of PLD activity (Figs. 2b,c). However, in the presence of Ca^{2+}, GTP[S] with and without PMA produced a full secretory response that was associated with maximal PA formation. Progressive inhibition of PA formation by increasing ethanol levels was associated with parallel increases in PEt, but had no effect on secretion. Since the concentration of PA formed in response to GTP[S] (both with and without PMA) in the presence of 400 mM-ethanol was comparable to that formed by PMA in the absence of ethanol (Fig. 2b), the results suggest that the strong stimulation of PLD by GTP[S] and Ca^{2+} still results in the formation of sufficient PA to support secretion. Although the GTP[S]-induced formation of DAG itself, or of PA derived from DAG, did not correlate with secretion, it is possible that this DAG, perhaps in conjunction with PEt [35,37], might replace PA as an activator of PKC, thereby further reducing the concentration of PA required for the stimulation of secretion by another mechanism, perhaps through the activation of a protein phosphatase [38]. In the presence of Ca^{2+}, PMA could replace DAG in such a model and thus, in combination with GTP[S], would produce effects on PLD activity and secretion similar to those seen with GTP[S] alone (Fig. 2). Furthermore, in the absence of Ca^{2+}, PMA can fully support PKC activity [6], and thus all PA formed by the synergistic combination of GTP[S] and PMA could be directly involved in

the stimulation of secretion. Thus, even in the presence of Ca^{2+}, the PA formed by PLD appears to be associated with at least 50% of the secretory response observed in the presence of GTP[S] and/or PMA.

GTP[S] and PMA dose-response experiments in the absence of Ca^{2+} further supported a role for PA produced by PLD in the control of secretion. Increasing concentrations of GTP[S], in combination with 10 and 100 nM PMA produced progressive increases in PLD activity that were associated with parallel increases in 5-HT secretion. These data confirm that secretion is only associated with PA formed by PLD; basal formation of PA was from another source not inhibited by ethanol and was not involved in secretion. The rightward shift in the relationship between PEt or PA formation and the secretion of 5-HT in the presence of 100 nM PMA, relative to that seen with 10 nM PMA, suggests (i) an inhibitory component of PMA activity that affects secretion; (ii) the presence of a second PLD activity that is enhanced by PKC but not associated with secretion; or (iii) the promotion of secretion at low PMA concentrations by a mechanism that does not involve PLD. Since PKC activity is not essential to GTP[S]-induced secretion in the presence of Ca^{2+} [35], another effect of low PMA concentrations seems unlikely, but cannot be fully excluded. Furthermore, studies involving the fusion of phospholipid vesicles have identified an inhibitory action of PMA on membrane fusion [39] and a recent report [40] has also identified two separate PLD activities in Madin-Darby canine

kidney cells. Thus, in the absence of more specific information regarding the substrates of human platelet PLD or secondary effects of low PMA concentrations. the apparent inhibitory effects are most simply interpreted as the result of nonspecific membrane effects at the higher dose of PMA.

The time-course analysis also showed that PLD activity correlated with secretion induced by GTP[S], in the absence and presence of PMA. Although the formation of [^{3}H]PA appeared to be slightly slower than the secretion of [^{14}C]5-HT, particularly at early time points (0-5 min), inhibitors of both lipoxygenase and cyclooxygenase pathways, as well as a potent 5-HT receptor antagonist, had no effect on secretion or PLD activity, indicating that PLD was stimulated independently of secretion. In this respect, complete inhibition of a Ca^{2+}-independent platelet PLA_2 activity that we have recently identified also had no effect on secretion or PLD activity (Coorssen and Haslam, unpublished observations). The results over the time-course analyzed are consistent with a small local formation of PA, by PLD, that is coupled with secretion; however, unlike secretion, the PLD activity appears to continue even after the exocytotic event. *In vivo*, such a prolonged PLD activity at a site of vascular trauma, subsequent to platelet activation and adhesion, would be consistent with a mitogenic role for PA [41] that could promote wound repair.

Together with our previous studies which demonstrated that the Ca^{2+}-chelator BAPTA and its analogues caused

comparable dose-dependent inhibitions of both secretion and PLD activity in the absence or presence of Ca^{2+} [25,35], the results presented here lend further support to the concept that PLD activity is an important regulator of secretion. A similar role for PLD has also been suggested in mast cells [20], HL-60 cells [22] and pancreatic islets [21]. We have previously proposed that PLD might be the target of G_E in the platelet and a similar conclusion has recently been reached regarding PLD in human neutrophils [42]. Evidence for a role of PA in platelet activation has also been obtained from studies involving supplementation with exogenous PA [43]. However, although secretion correlates with the formation of PA (and PEt) in intact thrombin-stimulated human platelets, PLD accounts for only 10 - 20% of the total PA produced [24,35]. Therefore, a major role for PLD in secretion from platelets is only possible if the PA produced by this enzyme differs from that generated by the sequential actions of PLC and DAG-kinase. In this respect, permeabilized platelets appear to be different from intact platelets in that PLD appears to be the principal source of PA in the former, and that PA formed via the DAG-kinase pathway is not associated with secretion, even in the presence of Ca^{2+}. The other product of PLD activation, a headgroup moiety (probably choline), is unlikely to serve in a second messenger capacity to mediate secretion since addition of various "headgroups" and "phospho-headgroups" (PLD and PLC products, respectively) had no effect on secretion from permeabilized platelets

(Coorssen and Haslam, unpublished observations).

The PA produced by PLD may in fact be distinct in either its fatty acid composition or in its subcellular localization, a conclusion supported by at least two lines of evidence. First, secretion from permeabilized platelets correlates only with the PA formed in response to the stimulation of PLD. Second, exogenous PLD, which can only affect the external monolayer of the plasma membrane in electropermeabilized platelets, produces substantial increases in the total PA content of stimulated platelets but there is no corresponding enhancement of 5-HT secretion (Coorssen and Haslam, unpublished observations). This latter result strongly suggests a distinct subcellular localization of the PA produced by endogenous PLD. A PLD activity has previously been associated with the azurophilic granules of human neutrophils [44], the secretion of which is known to be regulated by G_E [45]. Together, these results suggest the possibility that PA produced in granule membranes is an important mediator of the secretory process. This PA could enhance secretion by several mechanisms including the stimulation of PKC [35,46] or other kinases [36], regulatory effects on factors that control the activities of low-M_r GTP-binding proteins [47], the promotion of annexin binding to specific membrane sites [48] as well as a direct membrane-fusogenic action [49,50].

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Fig. 1. Effects of different ethanol concentrations on Ca^{2+}-independent secretion and on the associated formation of [^{3}H]PA and [^{3}H]PEt

Samples of labelled permeabilized platelets were equilibrated and incubated at pCa > 9 with no stimulus (○) or with 100 μM-GTP[S] and 100 nM-PMA (■), in each case in the presence of the indicated concentrations of ethanol. The secretion of [^{14}C]5-HT *(a)* and the formation of [^{3}H]PA *(b)* and [^{3}H]PEt *(c)* were determined in the same samples. Values are means ± S.E. from triplicate incubations.

Fig. 2. Effects of different ethanol concentrations on secretion and on the formation of [^{3}H]PA, [^{3}H]PEt and [^{3}H]DAG in the presence of Ca^{2+}

Samples of labelled permeabilized platelets were equilibrated and incubated at a pCa of 6 with no stimulus (○), 100 μM-GTP[S] (▼), 100 nM-PMA (▲) or 100 μM-GTP[S] and 100 nM-PMA (■), in each case in the presence of the indicated concentrations of ethanol. The secretion of [^{14}C]5-HT *(a)* and the formation of [^{3}H]PA *(b)*, [^{3}H]PEt *(c)* and [^{3}H]DAG *(d)* were determined in the same samples. Values are means ± S.E. from triplicate incubations.

Fig. 3. Relationships between the activation of PLD and Ca^{2+}-independent [^{14}C]5-HT secretion in permeabilized platelets incubated with different concentrations of GTP[S] and PMA

Samples of labelled permeabilized platelets were equilibrated and incubated with the indicated concentrations of GTP[S], both in the absence of PMA (▼) and in the presence of 10 nM-PMA (●) or 100 nM-PMA (■). Incubations were carried out in both the presence and absence of 200 mM-ethanol. The secretion of [^{14}C]5-HT and the formation of [^{3}H]PEt and [^{3}H]PA were determined in these samples; values are means ± S.E. from triplicate incubations. *a*, concentration-response curves for the effects of GTP[S] on [^{14}C]5-HT secretion in the presence of 200 mM-ethanol; *b*, corresponding concentration-response curves for the effects of GTP[S] on [^{3}H]PEt formation; *c*, relationships between [^{14}C]5-HT secretion and [^{3}H]PEt formation from the results shown in *(a)* and *(b)*; *d*, relationships between [^{14}C]5-HT secretion and [^{3}H]PA formation from incubations identical to those shown in *(a)* and *(b)*, apart from the omission of ethanol.

Fig. 4. Time course of Ca^{2+}-independent [^{14}C]5-HT secretion and [^{3}H]PA formation in permeabilized platelets stimulated by GTP[S] and/or PMA

Samples of labelled permeabilized platelets were equilibrated for 15 min at 0°C at pCa >9 with no stimulus, 100 μM-GTP[S] (▼), 100 nM-PMA (▲) or 100 μM-GTP[S] and 100 nM-PMA (■), each in the presence of 200 mM-ethanol. Following equilibration, samples were incubated at 25°C for the times indicated. The secretion of [^{14}C]5-HT *(a)* and the formation of [^{3}H]PA *(b)* were determined in the same samples. The [^{3}H]PA formed in control (no stimulus) samples was not associated with the secretion of any [^{14}C]5-HT, or with the formation of any [^{3}H]PEt in parallel samples containing 200 mM-ethanol. The concentrations of [^{3}H]PA in control samples were therefore subtracted from the concentrations of [^{3}H]PA determined in the stimulated samples, to show absolute increases in [^{3}H]PA formed by PLD. Values are means ± S.E. from triplicate incubations.

Fig. I

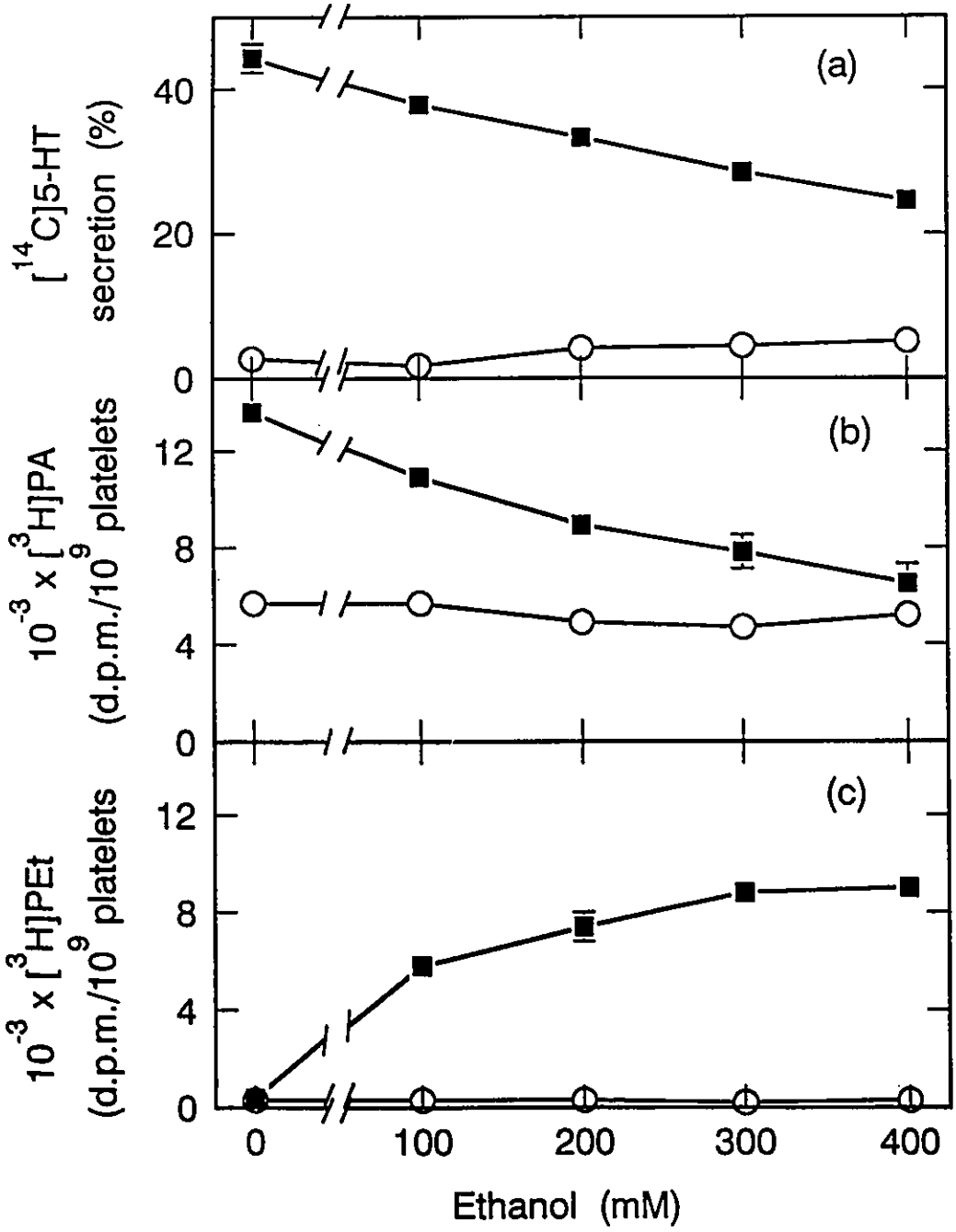
(a)
(b)
(c)
40
20
0
12
8
4
0
12
8
4
0
0
100
200
300
400
Ethanol (mM)
[14C]5-HT secretion (%)
10-3 x [3H]PA (d.p.m./10^9 platelets
10-3 x [3H]PEt (d.p.m./10^9 platelets

Fig. 2

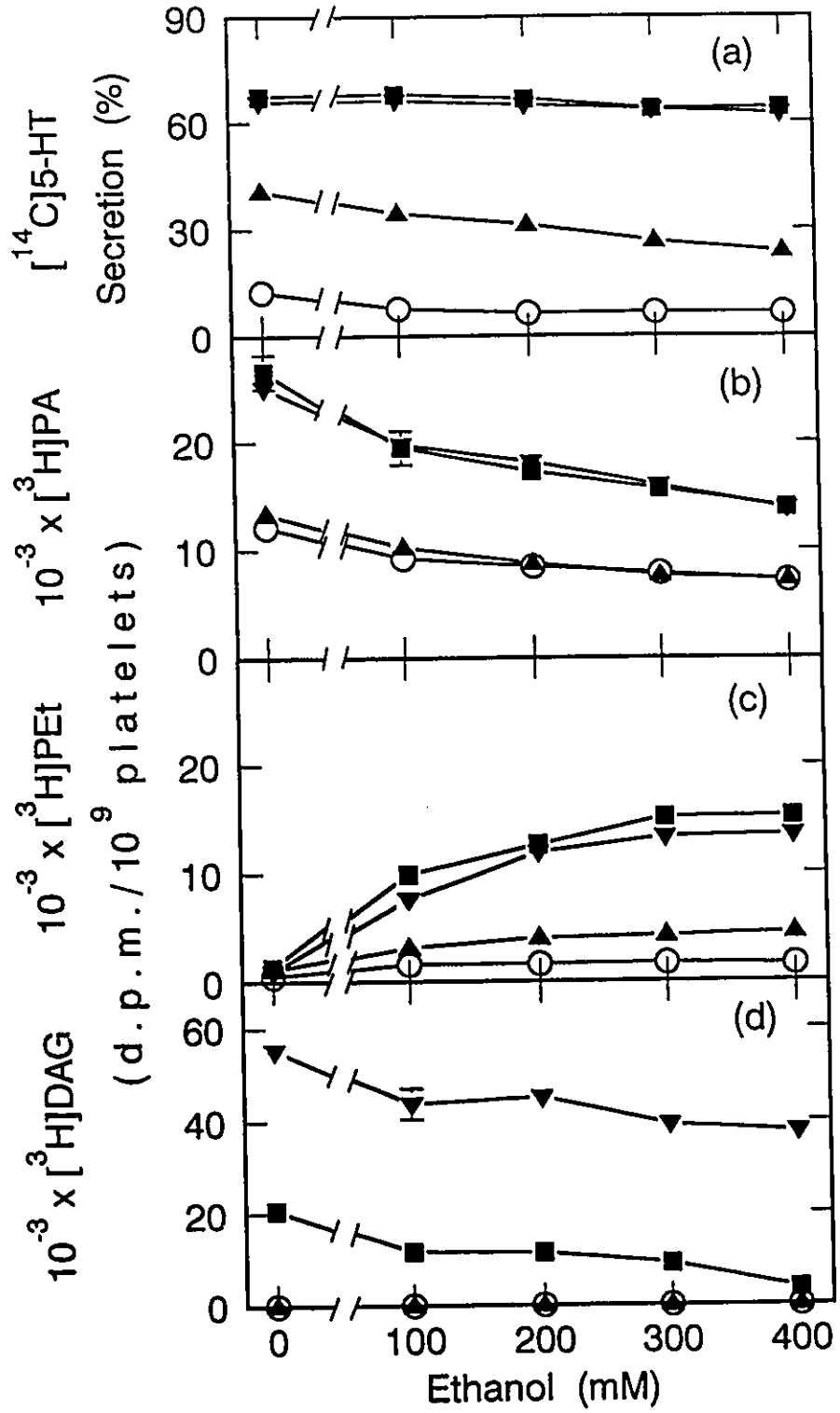
[14C]5-HT Secretion (%)
10-3 x [3H]PA
10-3 x [3H]PEt
10-3 x [3H]DAG
(d.p.m./10^9 platelets)
(a)
(b)
(c)
(d)
90
60
30
0
20
10
0
20
10
0
60
40
20
0
0
100
200
300
400
Ethanol (mM)

Fig. 3

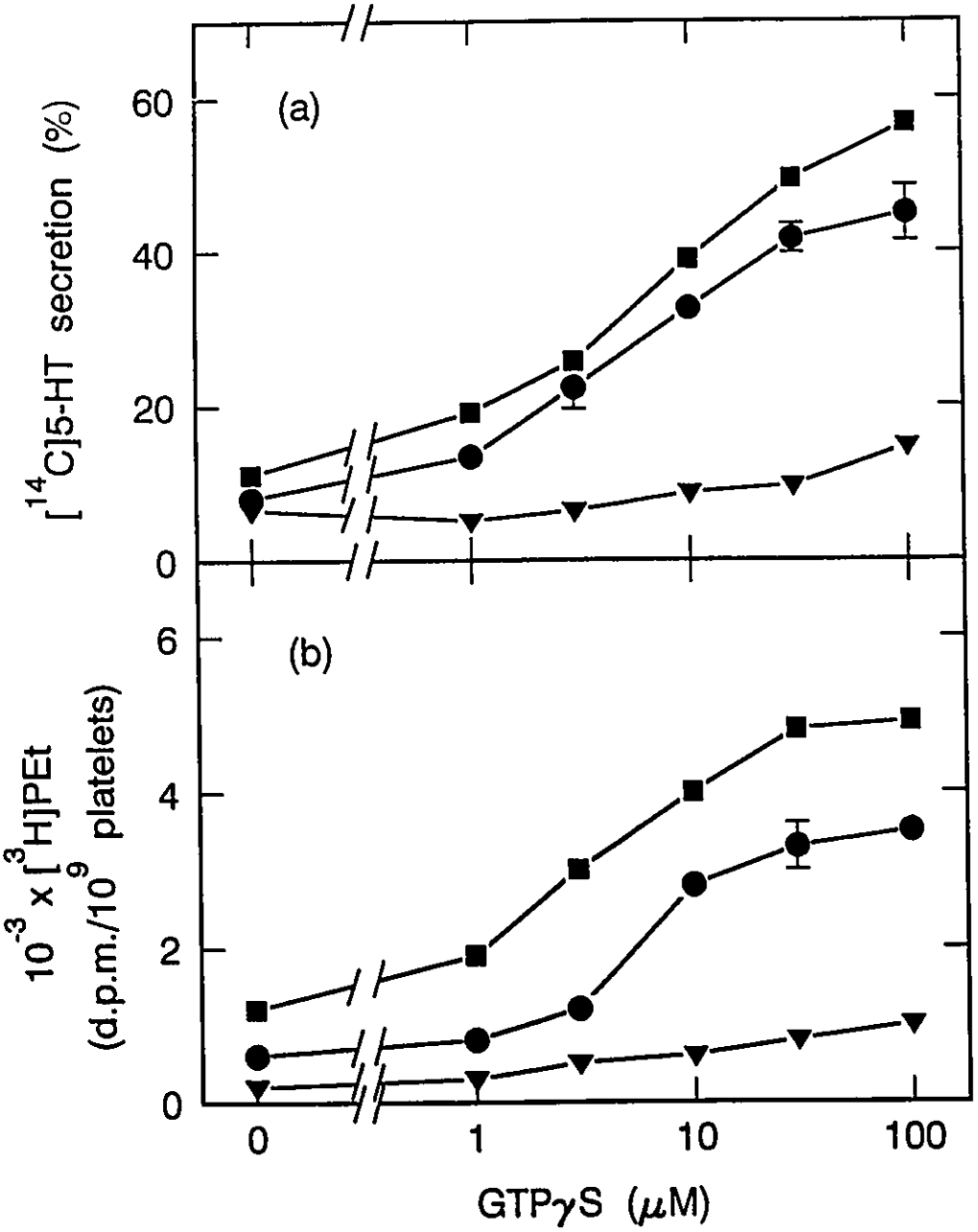
(a)
60
40
20
0
[^{14}C]5-HT secretion (%)
(b)
6
4
2
0
10^{-3} x [^{3}H]PEt
(d.p.m./10^{9} platelets)
0
1
10
100
GTPγS (μM)

Fig. 3

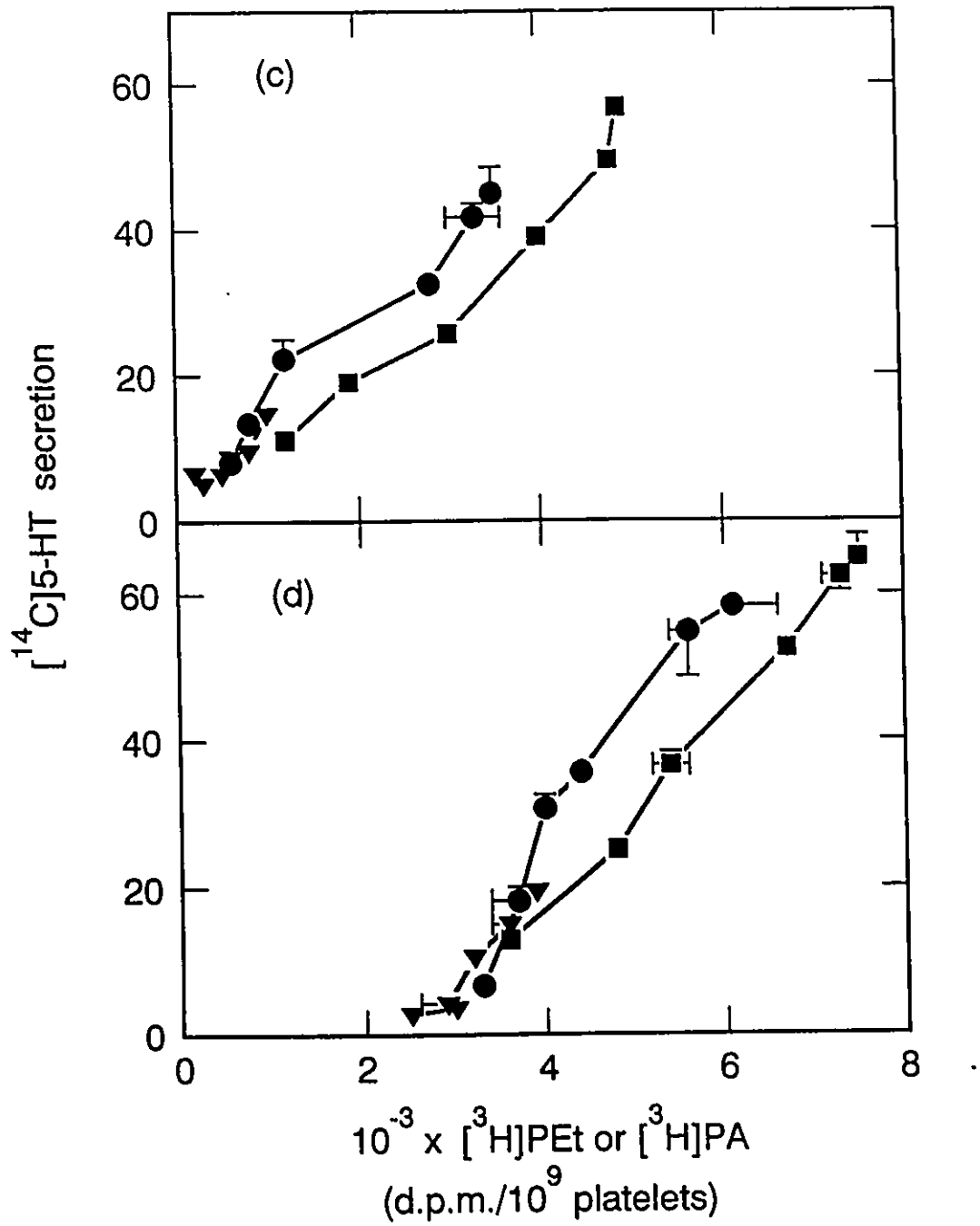
(c)
(d)
60
40
20
0
60
40
20
0
0
2
4
6
8
[14C]5-HT secretion
10-3 x [3H]PEt or [3H]PA
(d.p.m./10^9 platelets)

Fig. 4

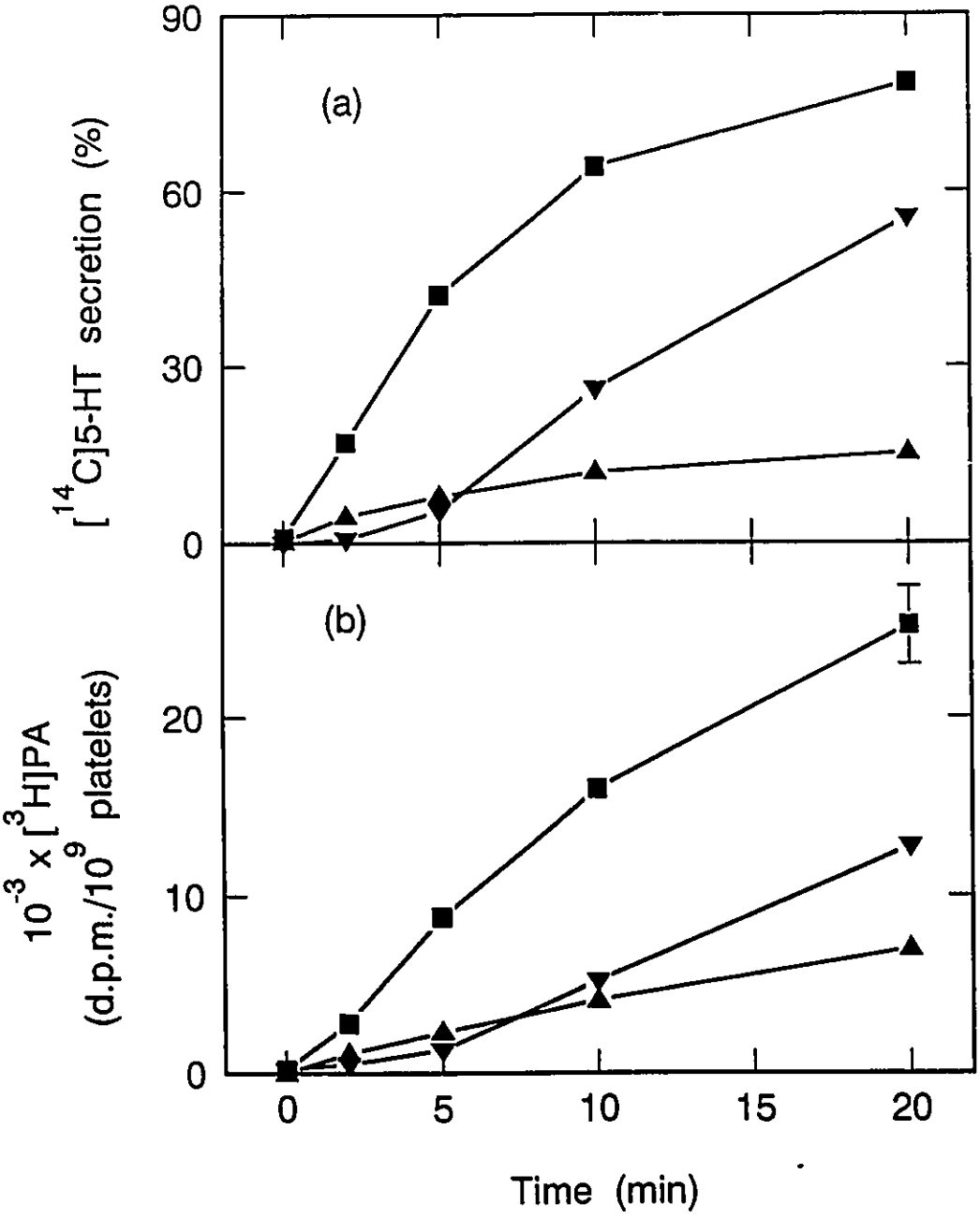
90
60
30
0
(a)
[^{14}C]5-HT secretion (%)
(b)
20
10
0
10^{-3} x [^{3}H]PA
(d.p.m./10^{9} platelets)
0
5
10
15
20
Time (min)

Table 1. Effects of GTP[S], PMA and Ca^{2+} on dense granule secretion and on the accumulation of [^{3}H]DAG in permeabilized platelets

Samples of permeabilized platelets containing dense granule [^{14}C]5-HT and phospholipids labelled with [^{3}H]arachidonate were equilibrated (15 min at 0°C) and incubated (10 min at 25°C) with the indicated combinations of GTP[S] (100 μM) and PMA (100 nM) in either the absence of Ca^{2+} (pCa > 9) or the presence of Ca^{2+} (pCa 6). The secretion of [^{14}C]5-HT and the formation of [^{3}H]DAG were measured. The initial amount of [^{3}H]DAG in the platelet suspension (6.0 ± 0.2 x 10^3 d.p.m./10^9 platelets) was subtracted in each case to determine the net change in [^{3}H]DAG. Values are means ± S.E. from triplicate samples in the same experiment.

Additions	Secretion of [^{14}C]5-HT (%)	Change in [^{3}H]DAG (10^{-3} x d.p.m./10^9 platelets)
None	1 ± 0	-2.5 ± 0.2
GTP[S]	19 ± 1	-1.6 ± 0.2
PMA	11 ± 0	-2.4 ± 0.2
GTP[S] + PMA	51 ± 1	-1.6 ± 0.3
Ca^{2+}	10 ± 1	-1.2 ± 0.4
Ca^{2+} + GTP[S]	72 ± 3	+28.0 ± 1.6
Ca^{2+} + PMA	48 ± 2	-1.7 ± 0.2
Ca^{2+} + GTP[S] + PMA	73 ± 2	+7.4 ± 0.3

Table 2. Relationships between dense granule secretion from permeabilized platelets incubated with GTP[S], PMA and Ca^{2+} and the formation of [^{3}H]PA and [^{3}H]PEt in the absence and presence of ethanol

Samples of permeabilized platelets containing dense granule [^{14}C]5-HT and phospholipids labelled with [^{3}H]arachidonate were equilibrated and incubated with the indicated combinations of GTP[S] (100 μM), PMA (100 nM) and ethanol (200 mM), in both the absence and presence of Ca^{2+} (pCa > 9 and 6). Secretion of [^{14}C]5-HT and the accumulation of [^{3}H]PA and of [^{3}H]PEt were determined; values are means ± S.E. from triplicate samples in the same experiment.

Additions	Ethanol	Secretion of [^{14}C]5-HT (%)	Formation of [^{3}H]phospholipid metabolites (10^{-3} x d.p.m./10^9 platelets)	
			[^{3}H]PA	[^{3}H]PEt
None	-	4 ± 1	2.0 ± 0.3	0.0 ± 0.1
	+	6 ± 1	2.2 ± 0.3	0.1 ± 0.1
GTP[S]	-	19 ± 1	3.8 ± 0.3	0.1 ± 0.1
	+	14 ± 1	2.5 ± 0.3	1.3 ± 0.1
PMA	-	14 ± 2	3.2 ± 0.4	0.1 ± 0.1
	+	11 ± 0	2.5 ± 0.3	1.6 ± 0.2
GTP[S] + PMA	-	71 ± 1	8.8 ± 0.3	0.2 ± 0.1
	+	60 ± 1	5.5 ± 0.3	9.6 ± 0.4
Ca^{2+}	-	9 ± 1	4.6 ± 0.5	0.3 ± 0.1
	+	9 ± 1	4.4 ± 0.3	1.1 ± 0.1
Ca^{2+} + GTP[S]	-	85 ± 1	16.1 ± 0.6	0.4 ± 0.1
	+	85 ± 2	10.2 ± 0.2	11.9 ± 0.5
Ca^{2+} + PMA	-	53 ± 3	7.2 ± 0.4	0.2 ± 0.1
	+	44 ± 2	4.1 ± 0.3	3.6 ± 0.4
Ca^{2+} + GTP[S] + PMA	-	88 ± 1	18.9 ± 2.2	0.3 ± 0.1
	+	85 ± 2	12.4 ± 1.2	14.2 ± 0.5

PAPER 4

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GTPγS and phorbol ester act synergistically to stimulate both Ca^{2+}-independent secretion and phospholipase D activity in permeabilized human platelets

Inhibition by BAPTA and analogues

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We have tested the hypothesis that phospholipase D (PLD) is the effector of the unidentified G protein (G_E) mediating Ca^{2+}-independent exocytosis in platelets. Although GTPγS, and to a lesser extent phorbol 12-myristate 13-acetate (PMA), caused some secretion of 5-HT from electropermeabilized human platelets in the effective absence of Ca^{2+} (pCa > 9), these stimuli had much more potent synergistic effects when added together. In all cases, secretion of 5-HT was closely correlated to the stimulus-induced formation of [^{3}H]phosphatidic acid ([^{3}H]PA) from [^{3}H]arachidonate-labelled phospholipids. Addition of ethanol inhibited both secretion and [^{3}H]PA formation and led to the accumulation of [^{3}H]phosphatidylethanol ([^{3}H]PEt), indicating that [^{3}H]PA was formed largely by activation of PLD. BAPTA and analogues caused dose-dependent inhibitions of both GTPγS-induced secretion and PLD activity in the permeabilized platelets. This action of BAPTA did not appear to be mediated by chelation of Ca^{2+} or by direct inhibition of protein kinase C (PKC). The results suggest that PLD is the target of G_E in platelets and that BAPTA can block PLD activation.

GTPγS; Phosphatidic acid; Phospholipase D; BAPTA; Secretion; Permeabilized platelet

1. INTRODUCTION

A previous study from this laboratory [1] showed that GTPγS can induce the Ca^{2+}-independent secretion of both dense and α-granule constituents from electropermeabilized human platelets without causing stimulation of phosphoinositide-specific phospholipase C. This action of GTPγS was dependent on PKC activity [2] and was potentiated by phorbol ester (PMA) [1,2]. Similar studies with other permeabilized cells, particularly neutrophils [3], HL-60 cells [4] and RINm5F cells [5], in which Ca^{2+}-independent secretion was observed, and with mast cells in which Ca^{2+} was required [6,7], have led Gomperts and colleagues to propose that an unidentified G protein, designated G_E, may mediate GTPγS-induced exocytosis (reviewed in [8]). Since treatment of rabbit platelets with PMA enhances the ability of GTPγS to stimulate PLD in membrane preparations [9], we have suggested that PLD may be the effector of G_E in platelets [1]. In the present study, we have investigated this possibility and have demonstrated a close correlation between the Ca^{2+}-independent release of a dense granule constituent (5-HT) and activation of PLD in permeabilized human platelets incubated with GTPγS and/or PMA.

There is evidence that the Ca^{2+}-chelator BAPTA [10] can inhibit GTPγS-induced secretion from a number of cell types by a mechanism that may be unrelated to its ability to bind Ca^{2+} ions [11]. Thus in electropermeabilized platelets, the secretion of both dense granule and lysosomal constituents in response to GTPγS was abolished by 20 mM BAPTA but not by 20 mM EGTA, although the equilibrium affinities of these compounds for Ca^{2+} were similar under the conditions of these experiments [12]. In permeabilized neutrophils, much less GTPγS-induced secretion of β-glucuronidase was seen in the presence of BAPTA than of EGTA [3]. Finally, dibromo-BAPTA (4 mM) inhibited GTPγS-induced exocytosis from mast cells, despite the presence of Ca^{2+} [13]. Since these actions of BAPTA have not been adequately explained, we investigated the effects of BAPTA and analogues on both the Ca^{2+}-independent secretion of 5-HT from permeabilized platelets and the associated activation of PLD. The results provide further evidence of a role for PLD in secretion.

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Abbreviations: PLD, phospholipase D; GTPγS, guanosine 5'-[γ-thio]triphosphate; PMA, phorbol 12-myristate 13-acetate; PA, phosphatidic acid; PEt, phosphatidyl-ethanol; BAPTA, 1,2-bis(*o*-aminophenoxy)ethane-*N*,*N*,*N*',*N*'-tetraacetic acid; PKC, protein kinase C

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2. EXPERIMENTAL

2.1. *Materials*

[5,6,8,9,11,12,14,15-^{3}H]Arachidonic acid (100 Ci/mmol) and [γ-^{32}P]ATP (3000 Ci/mmol) were obtained from Du Pont, Mississauga, ON, Canada, and [*side-chain*-2-^{14}C]5-HT (55 mCi/mmol) from Amersham, Oakville, ON, Canada. GTPγS was from Boehringer Mannheim, Laval, PQ, Canada, and PMA and BAPTA from Sigma, St. Louis, MO, USA. BAPTA was also obtained from Fluka, Ronkonkoma, NY, USA. 5,5'-Dibromo-BAPTA was from Molecular Probes, Eugene, OR, USA and 5,5'-dinitro-BAPTA was a generous gift from Dr. M.P. Charlton, Department of Physiology, University of Toronto, Toronto, ON, Canada. Other materials were from sources listed previously [1,14].

2.2. *Labelling and electropermeabilization of platelets*

Human platelets, isolated as described previously [14], were resuspended at 5×10^8/ml in citrated plasma, pH 6.5, and incubated at 37°C for 1.5 h with [^{3}H]arachidonate (5 μCi/ml). [^{14}C]5-HT was added in three equal amounts during the last 30 min (to a final concentration of 0.25 μCi/ml). The platelets were then washed in Ca^{2+}-free Tyrode's solution containing 5 mM PIPES (pH 6.5) and 0.35% (w/v) bovine serum albumin and, after addition of 5 mM EGTA, were permeabilized by high-voltage electric discharges [14]. The permeabilized platelets were cooled to 4°C, freed from low-M_r solutes by gel filtration [14] and eluted in a medium (pH 7.4) containing 3.9 mM $MgCl_2$ and the potassium salts of glutamic acid (160 mM), HEPES (20 mM), EDTA (2.5 mM) and EGTA (2.5 mM). ATP (final concentration 5 mM) was added to the eluate, which was adjusted to contain 5×10^7 platelets/ml. This suspension was stored at 0°C until used (30–60 min).

2.3. *Incubations*

All samples of permeabilized platelet suspension were first equilibrated for 15 min at 0°C with an additional 10 mM EGTA (to give a pCa > 9 even after release of platelet Ca^{2+} [1]), with sufficient $MgCl_2$ to give 5.0 mM Mg^{2+}_{free} and with any other additions. Concentrations of added $MgCl_2$ were calculated using binding constants given elsewhere [10,15]. After equilibration, samples (final vol. 0.4 ml containing 1.6×10^8 platelets) were incubated for 10 min at 25°C before measurement of [^{14}C]5-HT secretion in 0.05 ml portions [14] and extraction of lipids from the remainder [16]. [^{3}H]PA and [^{3}H]PEt were then isolated by t.l.c. [17] and counted for ^{3}H as in [9]. Basal values for extracellular [^{14}C]5-HT and for ^{3}H-labelled lipids were obtained from permeabilized platelet suspension stored at 0°C and subtracted from values obtained after incubations. These corrections were very small for [^{14}C]5-HT and [^{3}H]PEt, but significant [^{3}H]PA was present prior to incubations. To normalize results from different experiments, ^{3}H-labelled lipids were expressed as percentages of total platelet ^{3}H. In some experiments, PKC activation was measured in small samples of permeabilized platelet suspension (0.1 ml) containing [γ-^{32}P]ATP (16 μCi) by determining the phosphorylation of its major substrate, pleckstrin [1,14].

3. RESULTS

3.1. *Relationship between secretion and [^{3}H]PA formation*

In the absence of additions, incubation of permeabilized platelets at a pCa > 9 did not lead to [^{14}C]5-HT secretion, though some [^{3}H]PA was formed (Fig. 1). However, equilibration of the platelets with 100 μM GTPγS, or even with 100 nM PMA, caused significant secretion of [^{14}C]5-HT at pCa > 9 and enhanced [^{3}H]PA formation. Moreover, when added together, these stimuli induced a much more marked secretion that was on average 2.2-fold greater than the sum of their individual effects (Fig. 1A). In these experiments, the effect of

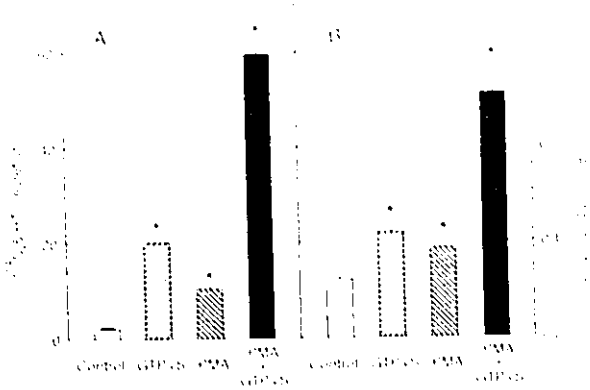

Fig. 1. Effects of GTPγS and PMA on Ca^{2+}-independent 5-HT secretion and PA formation in permeabilized platelets. Samples of labelled permeabilized platelets were incubated with no stimulus (control), 100 μM GTPγS, 100 nM PMA or 100 μM GTPγS and 100 nM PMA, as indicated. Secretion of [^{14}C]5-HT (A) and the increases in [^{3}H]PA (B) were determined. To normalize results from different experiments, [^{3}H]PA was expressed as a percentage of the total ^{3}H in the permeabilized platelets; basal [^{3}H]PA (0.071 ± 0.004% of platelet ^{3}H) was subtracted. Values shown are means ± S.E. from all experiments ($n = 17$); the significance of changes was evaluated by two-sided paired t-tests (*$P < 0.001$).

PMA alone was weaker and the synergism between GTPγS and PMA stronger than in a previous study at pCa > 9 [1] in which the $[Mg^{2+}_{free}]$ was 2.7 mM, rather than 5.0 mM. Measurement of [^{3}H]PA accumulation in the same experimental samples (Fig. 1B) showed that the stimulus-induced formation of this phospholipid closely paralleled secretion. In particular, the increases in [^{3}H]PA caused by simultaneous addition of GTPγS and PMA amounted to 2.4-fold the sum of the increases they induced individually.

3.2. *Role of PLD activity in [^{3}H]PA formation*

Since phosphoinositide-specific phospholipase C activity was not detected in permeabilized platelets incubated at pCa > 9 [1], the role of PLD in [^{3}H]PA formation was evaluated. The decreases in the amounts of [^{3}H]PA formed on addition of ethanol and the associated accumulation of [^{3}H]PEt by transphosphatidylation were used as measures of PLD activity [17]. Addition of 400 mM ethanol markedly inhibited the accumulation of [^{3}H]PA caused by GTPγS alone or by GTPγS and PMA (Fig. 2A); in three separate experiments, these inhibitions amounted to 64 ± 7% and 74 ± 7%, respectively (mean values ± S.E.). These actions of ethanol were associated with the formation of amounts of [^{3}H]PEt comparable to or larger than the decreases in [^{3}H]PA (Fig. 2B). When present together, GTPγS and PMA had markedly supra-additive effects on [^{3}H]PEt formation (Fig. 2B), showing that these stimuli acted synergistically to increase PLD activity. Although ethanol had little effect on [^{3}H]PA accumulation in platelets incubated with PMA alone, [^{3}H]PEt formation was al-

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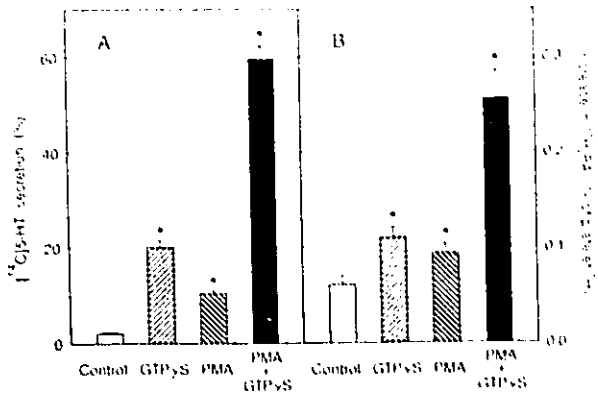

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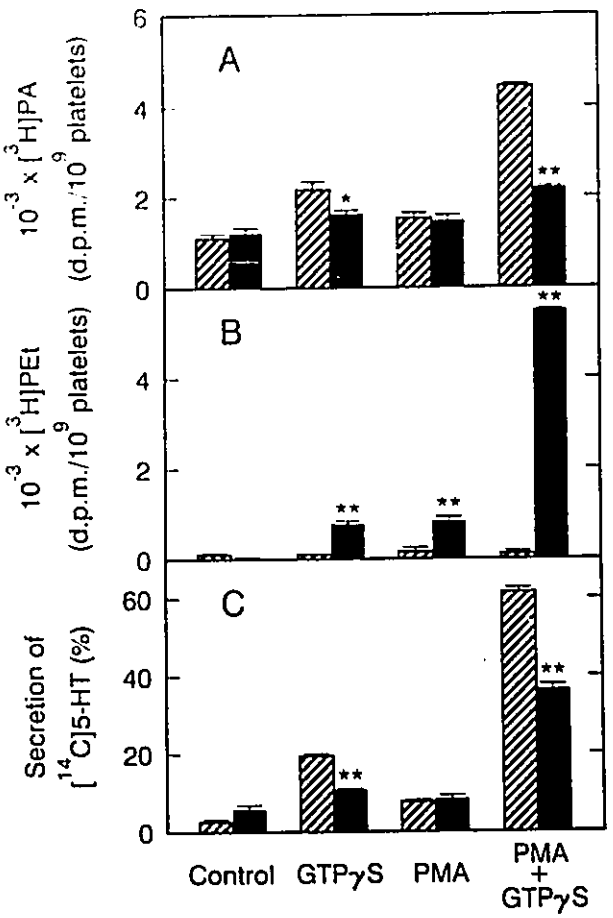

Fig. 2. Effects of ethanol on Ca^{2+}-independent PA and PEt formation and on the associated 5-HT secretion. Samples of labelled permeabilized platelets were incubated with no stimulus (control), 100 μM GTPγS, 100 nM PMA or 100 μM GTPγS and 100 nM PMA, in each case in the absence (▨) and presence (■) of 400 mM ethanol. The formation of both [^{3}H]PA (A) and [^{3}H]PEt (B) and the secretion of [^{14}C]5-HT (C) were determined. Values are means ± S.E. from triplicate incubations in a single experiment; significant effects of ethanol (two-sided unpaired *t*-test) are shown: $^*P < 0.05$; $^{**}P < 0.01$.

ways observed. However, in control incubations with ethanol, little or no [^{3}H]PEt was detected and ethanol tended to increase rather than decrease [^{3}H]PA accumulation, though this was rarely significant in individual experiments (Fig. 2A). These results indicate that stimulus-induced [^{3}H]PA formation is largely attributable to PLD activity, whereas in control incubations, [^{3}H]PA appears to be formed by other mechanisms.

Ethanol (400 mM) markedly inhibited [^{14}C]5-HT secretion induced by GTPγS or by GTPγS and PMA (Fig. 2C): these inhibitions amounted to 74 ± 3% and 47 ± 3%, respectively (means ± S.E.; 3 experiments). However, ethanol had no significant effect on the weak secretion caused by PMA alone (Fig. 2C). This observation may, in part, be explained by a small increase in [^{14}C]5-HT secretion caused by ethanol in control samples. As a whole, these results demonstrate a correlation between the effects of ethanol on the accumulation of [^{3}H]PA attributable to PLD activation and its effect on [^{14}C]5-HT secretion. The residual secretion observed in the presence of ethanol correlated with both the residual stimulus-induced [^{3}H]PA accumulation and with [^{3}H]PEt formation.

3.3. *Inhibition of 5-HT secretion and PLD activation by BAPTA and analogues*

High (mM) concentrations of BAPTA have been reported to inhibit Ca^{2+}-independent secretion from permeabilized cells, including platelets [11,12]. The results in Table I confirm this and show that BAPTA also inhibited the increases in [^{3}H]PA caused by GTPγS and/or PMA. To study the relationship between the effects of BAPTA on secretion and PLD activity, [^{3}H]PEt formation was measured in the presence of 200 mM ethanol, which inhibited the secretion of [^{14}C]5-HT induced by GTPγS alone and by GTPγS with PMA by only 34 ± 4% and 15 ± 2%, respectively (mean values ± S.E., 5 experiments). Under these conditions, the IC_{50} for inhibition of the GTPγS-induced secretion of [^{14}C]5-HT

Table I

Effects of BAPTA on Ca^{2+}-independent 5-HT secretion, PA formation and pleckstrin phosphorylation in permeabilized platelets

Additions	Secretion of [^{14}C]5-HT (8%)		10^{-3} x Increase in [^{3}H]PA (d.p.m./10^9 platelets)		^{32}P in pleckstrin (pmol/10^9 platelets)	
	− BAPTA	+ BAPTA	− BAPTA	+ BAPTA	− BAPTA	+ BAPTA
None	3 ± 1	4 ± 0	3.3 ± 0.4	2.9 ± 0.4	101 ± 8	73 ± 6
GTPγS	26 ± 1	7 ± 1**	5.7 ± 0.3	3.6 ± 0.1**	219 ± 8	111 ± 2**
PMA	12 ± 1	8 ± 1*	5.2 ± 0.5	3.8 ± 0.1*	329 ± 3	320 ± 25
GTPγS + PMA	51 ± 3	25 ± 1**	12.3 ± 0.8	7.6 ± 0.5*	319 ± 9	326 ± 7

Samples of labelled permeabilized platelets were incubated with no stimulus, 100 μM GTPγS, 100 nM PMA or 100 μM GTPγS and 100 nM PMA, in each case in the absence and presence of 10 mM BAPTA. The secretion of [^{14}C]5-HT and formation of [^{3}H]PA were determined from triplicate incubations in the same experiment. [γ-^{32}P]ATP was added to additional duplicate samples for measurement of pleckstrin phosphorylation. Values are means ± S.E.; the significance of the effects of BAPTA was evaluated by two-sided unpaired *t*-tests ($^*P < 0.05$; $^{**}P < 0.01$).

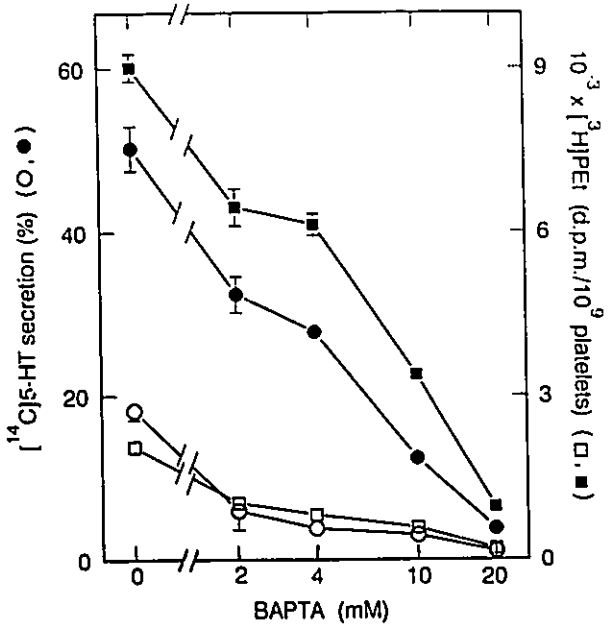

Fig. 3. Inhibition of Ca^{2+}-independent 5-HT secretion and PEt formation by BAPTA. Samples of labelled permeabilized platelets were incubated with the indicated concentrations of BAPTA and either 100 μM GTPγS (○, □) or 100 μM GTPγS and 100 nM PMA (●, ■), in each case in the presence of 200 mM ethanol. Secretion of [^{14}C]5-HT (○, ●) and the formation of [^{3}H]PEt (□, ■) were determined. Values are means ± S.E. from triplicate incubations in the same experiment.

by BAPTA was less than 2 mM, whereas the IC_{50} for inhibition of the synergistic effect of GTPγS and PMA was about 5 mM (Fig. 3). The results also show that BAPTA caused parallel dose-dependent inhibitions of PLD activation ([^{3}H]PEt formation). In five separate experiments, 10 mM BAPTA inhibited GTPγS-induced secretion and [^{3}H]PEt formation by 71 ± 3% and 64 ± 5%, respectively, whereas the corresponding effects of GTPγS with PMA were inhibited by 66 ± 3% and 52 ± 3%, respectively (mean values ± S.E.). Dibromo-BAPTA was considerably more effective than BAPTA as an inhibitor of both the Ca^{2+}-independent [^{14}C]5-HT secretion and [^{3}H]PEt formation induced by GTPγS with PMA; 1 mM dibromo-BAPTA and 4 mM BAPTA were roughly equipotent (Table II). Dinitro-BAPTA had effects on secretion and PLD activity intermediate between those of BAPTA and dibromo-BAPTA (not shown).

To investigate the mechanism of action of BAPTA, the phosphorylation of pleckstrin during Ca^{2+}-independent secretion from permeabilized platelets was also studied (Table I). BAPTA (10 mM) did not affect the phosphorylation of this protein induced by PMA added with or without GTPγS, indicating that BAPTA did not directly inhibit PKC. However, BAPTA did inhibit the phosphorylation of pleckstrin caused by GTPγS alone, suggesting that PA formed by PLD, or a PA metabolite, may activate PKC.

4. DISCUSSION

The results provide three independent lines of evidence consistent with the hypothesis [1] that the target of G_i in the platelet is PLD. First, GTPγS and PMA acted synergistically in permeabilized platelets to stimulate both Ca^{2+}-independent secretion of 5-HT and PLD activity, whether the latter was measured as the accumulation of [^{3}H]PA in the absence of ethanol or of [^{3}H]PEt in the presence of ethanol. This synergistic activation of PLD is in accord with a previous report in which rabbit platelet membranes were studied [9], as well as with recent work on permeabilized HL-60 cells [18]. Second, ethanol inhibited the GTPγS-induced secretion of 5-HT from permeabilized platelets and caused comparable decreases in [^{3}H]PA accumulation, which were attributable to the formation of [^{3}H]PEt by PLD-catalysed transphosphatidylation. Similar effects of ethanol on stimulus-induced PA accumulation and secretion in intact mast cells [19] and differentiated HL-60 cells [20] have been cited as evidence of a role for PLD in these cells. Third, BAPTA and analogues caused parallel concentration-dependent inhibitions of GTPγS-induced secretion and PLD activity in permeabilized platelets, despite the presence of sufficient EGTA to give a pCa > 9. This unexpected result not only greatly strengthens the correlation between secretion and PLD activity, but can also explain previously reported anomalous inhibitory effects of BAPTA and analogues on secretion from permeabilized platelets [12] and other cells [3,11,13]. It is of interest that dibromo-BAPTA, which has an equilibrium affinity for Ca^{2+} about tenfold lower than that of BAPTA [10,21], was about fourfold more potent than BAPTA as an inhibi-

Table II

Comparison of the inhibitory effects of dibromo-BAPTA and BAPTA on Ca^{2+}-independent secretion and PLD activity in permeabilized platelets

Inhibitor	Secretion of [^{14}C]5-HT (%)	10^{-3} × Increase in [^{3}H]PEt (d.p.m./10^9 platelets)
None	41 ± 2	9.6 ± 0.4
Dibromo-BAPTA (1 mM)	27 ± 1*	7.9 ± 0.7
Dibromo-BAPTA (4 mM)	8 ± 1*	3.2 ± 0.1*
BAPTA (4 mM)	23 ± 1*	7.4 ± 0.2*

Samples of labelled permeabilized platelets were incubated with 100 μM GTPγS and 100 nM PMA in the presence of 200 mM ethanol and the indicated concentrations of dibromo-BAPTA or BAPTA. The secretion of [^{14}C]5-HT and formation of [^{3}H]PEt were determined from triplicate incubations in the same experiment; values are means ± S.E. The significance of changes was evaluated by two-sided unpaired t-tests (*$P < 0.05$).

tor of secretion and PLD activity. Although BAPTA binds Ca^{2+} more rapidly than does EGTA [10], which may account for the ability of the former to block neurotransmitter release [22], differences in the Ca^{2+}-binding kinetics of these chelating agents are unlikely to explain the present results. Thus, we have observed similar inhibitions of GTPγS-stimulated secretion and PLD activity by BAPTA in the presence of Ca^{2+} buffered to a final pCa of 6 [2]. Moreover, dinitro-BAPTA, which has little or no Ca^{2+}-binding capacity [21], was slightly more potent than BAPTA as an inhibitor of secretion and PLD activity in our experiments. We conclude that BAPTA and analogues do not inhibit secretion by chelation of Ca^{2+}. In addition, BAPTA did not appear to exert a direct inhibitory effect on PKC in permeabilized platelets and may therefore act on either G_i or PLD itself.

Although our results are most simply explained if activation of PLD by a hypothetical G_R mediates the stimulation of secretion by GTPγS, more complex mechanisms involving multiple GTP-binding proteins and target enzymes cannot easily be excluded, particularly in intact cells. However, the absence of significant GTPγS-induced inositol phosphate or diacylglycerol formation in permeabilized platelets at pCa > 9 [1,2] indicates that phospholipase C activity has no role in secretion under the conditions of the present study. Moreover, although preliminary work shows that GTPγS and PMA act synergistically to stimulate phospholipase A_2 at pCa > 9, inhibition of this enzyme did not affect secretion from permeabilized platelets (Coorssen, Davidson and Haslam, in preparation). These considerations focus attention on the possibility that PA generated by PLD acts as a second messenger that mediates secretion from platelets. A similar role for PLD has also been suggested by others working with mast cells [19], HL-60 cells [20] and pancreatic islets [23]. However, in intact human platelets, PLD activity accounts for only 10–20% of the total PA that accumulates after stimulation by thrombin [2,24]; most of the PA is formed by the sequential actions of phospholipase C and diacylglycerol kinase. Thus, a major role for PLD in secretion from intact platelets is only likely if the PA generated by this enzyme differs in its fatty acid composition or, more plausibly, in its subcellular localization from that formed as a result of phospholipase C activity. Some support for this possibility is provided by our observation that the PA accumulating in control incubations of permeabilized platelets was formed by a PLD-independent mechanism and was not associated with secretion. There are multiple mechanisms through which PA generated by PLD might enhance secretion. These include activation of PKC, as suggested by the GTPγS-induced phosphorylation of pleckstrin [1], stimulation of other protein kinases [25], effects on factors that regulate low-M_r GTP-binding proteins [26] and a direct membrane-fusogenic action [27].

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PAPER 5

Phospholipase activation and secretion from permeabilized human platelets: Evidence that PLA_2 activity is not essential for secretion

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ABSTRACT

Although we have previously shown that strong correlations exist between phospholipase D (PLD)[1] activity and secretion from electropermeabilized platelets Coorssen and Haslam, 1993, *FEBS Lett.*, in the press), phospholipase A_2 (PLA_2) activity is also known to correlate with secretion in a number of cell types, including platelets. In order to investigate whether or not this enzyme might have a direct role in secretion, the effect of exogenous arachidonic acid (AA) on the secretion of dense granule [^{14}C]5-HT from electropermeabilized human platelets was studied. A variety of phospholipase inhibitors were tested on electropermeabilized platelets containing [^{3}H]AA-labelled phospholipids and dense granule [^{14}C]5-HT. Since we have previously established that secretion in platelets does not require the presence of Ca^{2+} ions and thus can occur in the absence of phospholipase C activity (Coorssen et al., 1990, *Cell Regulation 1*, 1027-1041), these studies were initially carried-out in the effective absence of free Ca^{2+} (pCa >9). Low concentrations of exogenous AA (10 - 40 μM) potentiated the GTP[S]-induced secretion of [^{14}C]5-HT. This action of AA was unaffected by inhibitors of AA metabolism (indomethacin or BW755C), indicating that eicosanoid formation was not responsible. At pCa >9, the endogenous formation of [^{3}H]AA correlated with the GTP[S]-induced secretion of [^{14}C]5-HT. PMA alone had no effect on [^{3}H]AA formation in the absence of Ca^{2+}, but could promote the effect of GTP[S], similarly to its effects on secretion. The presence of Ca^{2+} (pCa 6) also substantially enhanced the GTP[S]-induced formation of [^{3}H]AA.

Neither indomethacin or BW755C had any effect on secretion or on the Ca^{2+}-independent formation of [^{3}H]PA by phospholipase D (PLD). To establish whether or not PLA_2 activity is directly involved in the exocytotic mechanism, a variety of phospholipase inhibitors were tested at pCa >9 in the presence of BW755C. Certain compounds inhibited PLA_2 activity (NDGA, aristolochic acid and PMSF) but had no effect on PLD activity or secretion; only those compounds that affected PLD as well as PLA_2 activity (mepacrine and ethanol) inhibited secretion. Ethanol in particular inhibited Ca^{2+}-independent [^{3}H]PA accumulation and secretion but caused substantial enhancement of PLA_2 activity. The flavonoid quercetin also inhibited GTP[S]-induced PLA_2 and PLD activities, the phosphorylation of the endogenous PKC substrate pleckstrin and the secretion of dense granule 5-HT. However, in the presence of PMA, quercetin failed to inhibit GTP[S]-induced secretion despite inhibiting PLD activity. This ability of PMA to overcome the inhibitory effect of quercetin on GTP[S]-induced secretion and PKC activation suggests that the main role of the PA formed by PLD is to support the activation of PKC. This study shows for the first time that GTP[S] can induce a Ca^{2+}-independent PLA_2 activity in human platelets. However, this activity can be dissociated from secretion. The data are consistent with our previous hypothesis that activation of PLD may have an important role in secretion.

[1]Abbreviations: AA, arachidonic acid; BW755C, 3-amino-1-(3-trifluoromethylphenyl)-2-pyrazoline; GTP[S], guanosine 5'-o-(3-thiotriphosphate); 5-HT, 5-hydroxytryptamine; PA, phosphatidic acid; pCa, $-\log[Ca^{2+}_{free}]$; PEt, phosphatidylethanol; PKC, protein kinase C; PLA_2, phospholipase A_2; PLD, phospholipase D; PMA, phorbol 12-myristate 13-acetate; PMSF, phenylmethylsulfonyl fluoride; NDGA, nordihydroguaiaretic acid.

INTRODUCTION

The use of electropermeabilized preparations has contributed greatly to the identification of factors involved in the regulation of secretion from the three distinct secretory granule types found in platelets [1-6]. Earlier studies supported the concept that Ca^{2+} ions [1,2] and the guanine nucleotide-dependent activation of phosphoinositide-specific phospholipase C (PI-PLC), leading to the activation of PKC [2-4], were of major importance to the regulation of secretion. More recent studies [5,6] have shown that these factors have more modulatory effects rather than direct roles in the exocytotic mechanism. A study from this laboratory [5] established that GTP[S], a nonhydrolyzable analogue of GTP, could induce the Ca^{2+}-independent secretion of both dense and α-granule constituents from electropermeabilized human platelets, in the absence of PI-PLC activity. This action of GTP[S] was dependent on PKC activity [7] and was potentiated by phorbol ester (PMA) [5,7]. These findings were consistent with the existence of a particular G protein, designated G_E, that might mediate GTP[S]-induced secretion by regulating more terminal events in the exocytotic pathways of a number of different cell types [reviewed in 8 and 9]. We have recently shown that strong correlations exist between the activation of phospholipase D (PLD) and the secretion of dense granule constituents in both the absence and presence of Ca^{2+} [7,10], consistent with our previous hypothesis [5] that this enzyme may be the effector of G_E in platelets.

In the presence of Ca^{2+}, secretion also correlates with the

production of arachidonic acid (AA) via the stimulation of phospholipase A_2 (PLA_2) in platelets [11-13] as well as mast cells [14], chromaffin cells [15] and pancreatic islets [16]. The products of PLA_2 activation, a fatty acid (usually AA) and a lysophospholipid, have both been shown to act as membrane fusogens in model systems [17,18] and thus, by implication, could be directly involved in the final stage(s) of exocytosis. Recent studies have identified potent effects of fatty acids and lysophospholipids on the activation of PKC, including those species of the enzyme found in human platelets [19-21]. In addition, AA is largely converted to eicosanoids, including the potent platelet agonists prostaglandin H_2 and thromboxane A_2 [22]. Although increased PLA_2 activity appears to correlate with the secretion of granule contents from intact, thrombin-stimulated human platelets [13], the direct involvement of this enzyme in the exocytotic mechanism has not been established. Indeed, in some cell types, PLA_2 activation has been suggested as an absolute requirement for exocytosis [16], whereas in others it appears not to be involved [14,15].

Previous studies of guanine nucleotide-induced PLA_2 activity in permeabilized platelets [11,12] suggested that the enzyme was Ca^{2+}-dependent [9,10]. Furthermore, platelet α-granules are known to contain a species of small (~14 kDa) Ca^{2+}-dependent secretory (group II) PLA_2 [23]. Therefore, the correlations established between secretion and PLA_2 activity in studies to date, using intact and permeabilized platelets [11-13], may simply represent enzyme activity occurring subsequent to secretion. In this respect, the

secretion of α-granule constituents from intact platelets stimulated with exogenous AA was found to be specific for this fatty acid and to be dependent on its metabolism by a cyclooxygenase [24]. No study has yet shown a definite relationship between secretion and the cytosolic PLA_2 (group I) activity which is thought to be involved in signal transduction [25]. Several of these cytosolic PLA_2 species have now been isolated and characterized from a variety of cells including rat kidney [26], mouse spleen and a macrophage cell line [27], as well as from human [28], rabbit [29], sheep [30] and bovine [31] platelets. As a whole, these PLA_2 species share several common characterisitics including size (90 - 110 kDa), neutral pH optima and a preference for phosphatidylcholine, phosphatidylethanolamine and, in some cases phosphatidylserine, containing *sn*-2-arachidonoyl residues. Although these PLA_2 species are generally Ca^{2+}-dependent, that isolated from sheep platelets [30] did not require Ca^{2+} for activity. However, the cytosolic PLA_2 isolated from human platelets was Ca^{2+}-dependent [28] and no Ca^{2+}-independent PLA_2 activity has yet been characterized in human platelets [12]. Since our previous studies [5,7,10] established that Ca^{2+} (and thus PI-PLC activity) was not essential to the exocytotic mechanism, we thought it unlikely that a PLA_2 was the effector of G_E in human platelets [5]. Thus, although we have interpreted our results to date as indicative of a regulatory role for PA generated by PLD in the exocytotic mechanism, we have not directly proven that a cytosolic PLA_2 activity is not involved in secretion. Studies carried out in the effective absence of Ca^{2+} (pCa

>9) [5,10] have however largely eliminated any possible nonspecific effects of the secreted form of PLA_2, which is dependent on high (mM) Ca^{2+} concentrations. In the present study, we show for the first time that a Ca^{2+}-independent PLA_2 activity is not only present in human platelets but that it is stimulated by GTP[S], particulary in the presence of PMA. Although this was consistent with a role for PLA_2 in secretion, we found that this PLA_2 activity could be dissociated from the secretory response.

EXPERIMENTAL

Materials

[5,6,8,9,11,12,14,15-^{3}H]Arachidonic acid (100 Ci/mmol) and [γ-^{32}P]ATP (3000 Ci/mmol) were obtained from Du Pont (Mississauga, Ont., Canada) and [*side-chain*-2-^{14}C]5-HT (55 mCi/mmol) and [1-^{14}C]arachidonic acid (54.6 mCi/mmol) from Amersham (Oakville, Ont., Canada). GTP[S] was purchased from Boehringer Mannheim (Laval, Que., Canada) and arachidonic acid (Na^+-salt), aristolochic acid (52% Type I, 41% Type II), mepacrine, NDGA, PMA, PMSF and quercetin from Sigma (St. Louis, MO, U.S.A.). Quercetin was also obtained from Fluka (Ronkonkoma, NY, U.S.A.). Indomethacin was from Research Biochemicals Inc. (Natick, MA, U.S.A.). BW755C was a generous gift from Burroughs Wellcome Inc. (Beckenham, England). Other materials were from sources listed previously [2,5,10].

Labelling and electropermeabilization of platelets

Human platelets, isolated as previously described [2], were resuspended at 5 x 10^9/ml in citrated plasma (pH 6.5) and incubated with [^{3}H]arachidonate for 1.5 h at 37°C. [^{14}C]5-HT was added in three equal amounts (to a final concentration of 0.25 μCi/ml) during the last 30 min of labelling. The platelets were then washed in Ca^{2+}-free Tyrode's solution containing 5 mM-Pipes (pH 6.5), 0.35% (w/v) BSA, 50 units of heparin/ml and apyrase (30 μM/ml). After centrifugation, the platelets were resuspended in the same medium (without heparin or apyrase) and were permeabilized by high voltage electric discharges [2,5] after addition of 5 mM-EGTA. The permeabilized platelets were cooled to 4°C, freed from low-M_r solutes by gel filtration [2] and eluted in a medium (pH 7.4) containing 3.9 mM $MgCl_2$ and the potassium salts of glutamic acid (160 mM), Hepes (20 mM), EDTA (2.5 mM) and EGTA (2.5 mM). ATP (final concentration 5 mM) was added to the eluate, which was adjusted to contain 5 x 10^8 platelets/ml. This suspension was stored at 0°C until used (30-60 min).

Incubations

All samples of permeabilized platelet suspension (1.6 x 10^8 platelets) were first equilibrated at 0°C for 15 min with either an additional 10 mM-EGTA (pCa >9 even if all platelet Ca^{2+} were released [5]) or with sufficient $CaCl_2$ to give a pCa of 6. Specified additions, and sufficient $MgCl_2$ to give 5 mM-Mg^{2+}_{free}, were also added prior to equilibration; concentrations of $MgCl_2$ and $CaCl_2$ were

calculated using binding constants given elsewhere [32]. After equilibration, samples (0.4 ml final vol.) were incubated for 10 min at 25°C before measurement of [^{14}C]5-HT secretion in 0.05 ml portions [2] and extraction of lipids from the remainder using methanol/chloroform (2:1, by vol.) [33]. When both [^{3}H]AA and [^{3}H]PA were measured in the same experimental samples, extracts were divided into two equal volumes. [^{3}H]AA was isolated on one set of silica gel t.l.c. plates using a solvent containing hexane/diethyl ether/acetic acid (6:4:0.1, by vol.) [34] (Solvent 1); [^{3}H]PA and [^{3}H]PEt were isolated by t.l.c. in a solvent containing ethyl acetate/iso-octane/acetic acid (9:5:2, by vol.) [35]. Lipid migrations were determined using standards which were added to each sample prior to t.l.c. and were visualized by exposure to iodine vapour. Isolated lipids were scraped from the plates and counted for ^{3}H, as previously described [36]. When measured in separate experimental samples, [^{3}H]PA was extracted and isolated as described above, whereas samples for the determination of [^{3}H]AA were extracted with methanol/chloroform/HCl (2:1:0.3, by vol.) [37] and [^{3}H]AA was isolated by t.l.c. on silica gel plates using chloroform/methanol/acetic acid/water (90:8:1:0.8, by vol.) (Solvent 2) [38]. In some experiments a known amount of [^{14}C]AA (~300 d.p.m.) was included in each sample immediately prior to t.l.c. and the concentrations of [^{3}H]AA were determined after correction for the recovery of the [^{14}C]AA marker. Basal values for extracellular [^{14}C]5-HT and for ^{3}H-labelled lipids were obtained from permeabilized platelet suspension stored at 0°C; these basal values for [^{14}C]5-HT,

[^{3}H]PA and [^{3}H]PEt were always subtracted from values obtained after incubations. Unless otherwise noted, the same procedure was followed for [^{3}H]AA. These corrections were very small for [^{14}C]5-HT and [^{3}H]PEt, but significant [^{3}H]AA and [^{3}H]PA were present prior to incubations. In some experiments, the extent of PKC activation was determined in small samples of permeabilized platelet suspension (0.1 ml) containing [γ-^{32}P]ATP (16 μCi), by determining the phosphorylation of its major endogenous substrate, pleckstrin [2,3].

Data presentation

Each incubation condition was in triplicate in individual experiments, and each experiment was carried out at least two or three times. Values from representative experiments are given as means ± S.E. Pooled results from a number of experiments are given in the text as mean ± S.E.M. The significance of differences were determined by two-sided paired or unpaired *t* tests, as required.

RESULTS

Effects of exogenous AA on Ca^{2+}-independent secretion

We have previously established that Ca^{2+} is not an essential component of the exocytotic mechanism [5]. In this earlier study, GTP[S] (100 μM) and PMA (100nM) were each found to cause a limited secretion of dense granule [^{14}C]5-HT from electropermeabilized human platelets, but together these stimuli acted synergistically to cause a near maximal secretory response in the effective absence of Ca^{2+} (pCa >9). If AA does have a role in the exocytotic mechanism of

platelets it would also have to be produced and support secretion in the effective absence of Ca^{2+}. In the absence of AA, GTP[S] and PMA, added individually or simulataneously at pCa >9, caused [^{14}C]5-HT secretion to the extent reported previously (Table 1) [5,10]. The cyclooxygenase inhibitor indomethacin (20 μM) [39] and the dual cyclooxygenase/lipoxygenase inhibitor, BW755C (100 μM) [12,40,41], had no effects on these secretory responses (Table 1) indicating that metabolites of AA had no role under these conditions. To test for a possible effect of AA on the secretion of granule constituents from platelets, increasing concentrations of AA (Na^+-salt) were equilibrated and incubated with samples of permeabilized platelet suspension at pCa >9. In both the absence and presence of either indomethacin or BW755C, 10 μM-AA had no effect alone or in the presence of both GTP[S] and PMA but acted synergistically to enhance the GTP[S]-induced secretion of [^{14}C]5-HT (13 ± 2%; mean ± S.E., n=2). A much smaller but consistent enhancement of PMA-induced secretion was also observed (Table 1). More pronounced increases in GTP[S]-induced, and to a lesser extent PMA-induced secretion were observed with 40 μM-AA, which also caused some secretion of [^{14}C]5-HT under control conditions, particularly in the presence of either indomethacin or BW755C (Table 1). These increases in control secretion with 40 μM-AA could, to a large extent, account for the increases observed with GTP[S] and/or PMA. Since 100 μM-AA caused the complete release of [^{14}C]5-HT from permeabilized platelets (not shown), a lytic effect of high AA concentrations may account, in part, for the secretion of [^{14}C]5-HT caused by 40 μM-AA. However,

the enhancement of GTP[S]-induced secretion by 10 μM-AA suggests a specific potentiating effect of AA on the secretory mechanism. It is important to note that GTP[S]-induced PKC activity, which is essential for Ca^{2+}-independent secretion [7], was also enhanced by exogenous AA (not shown). In similar experiments, 100 μM-oleate (Na^+-salt) was required to enhance GTP[S]-induced secretion to the same extent as that observed with 10 μM-AA and 40 μM-oleate had no effect in either the absence or presence of GTP[S] or PMA. As a whole, these results suggested that fatty acids, in particular AA, could have a role in secretion, possibly by enhancing PKC activity or, at high local concentrations, by destabilizing membranes (a fusogenic effect).

Relationship between PLA_2 activity and secretion

To determine whether PLA_2 activity correlated with secretion, the formation of endogenous [^{3}H]AA was measured in permeabilized platelets in both the absence and presence of Ca^{2+}. Since the inhibitors indomethacin and BW755C did not cause any nonspecific effects on secretion (Table 1), these compounds were used to block the metabolism of [^{3}H]AA in order to obtain more accurate measurements of PLA_2 activity. As shown in Table 1, these compounds did not affect the limited Ca^{2+}-independent secretion caused by GTP[S] or PMA, or the near-maximal secretion caused by the synergistic combinations of GTP[S] and PMA or of GTP[S] and Ca^{2+} (Table 2). In the absence of indomethacin or BW755C, Ca^{2+}-independent formation of [^{3}H]AA was detected only after the addition of GTP[S] with PMA.

However, in the presence of the inhibitors small increases in [^{3}H]AA were detected in the presence of GTP[S] and PMA, which had no effect alone (Table 2). Thus, the formation of [^{3}H]AA correlated with the Ca^{2+}-independent secretion of [^{14}C]5-HT caused by GTP[S], in both the absence and presence of PMA, although PMA alone did not cause a detectable accumulation of [^{3}H]AA.

Ca^{2+} alone (pCa 6) did not increase the formation of [^{3}H]AA in these experiments, although a previous study [12] showed a substantial increase under similar conditions. In the presence of Ca^{2+}, GTP[S]-induced secretion of [^{14}C]5-HT was also unaffected by either indomethacin or BW755C, and both compounds caused marked enhancement of [^{3}H]AA accumulation in both the absence and presence of GTP[S] (Table 2). These results again indicated that AA metabolites did not affect the secretory mechanism. Moreover, the secretion of [^{14}C]5-HT also did not correlate with the accumulation of [^{3}H]AA. Thus, the results obtained at pCa 6 also indicated that AA was unlikely to have a direct membrane fusogenic effect in this system.

Since we have previously established that strong correlations exist between the Ca^{2+}-independent secretion of [^{14}C]5-HT and the formation of [^{3}H]PA by PLD [8], we sought to establish that the inhibitors of AA metabolism that were used in this study did not affect PA formation. BW755C (100 μM) did not affect the formation of [^{3}H]PA in control samples, or in the presence of GTP[S] and/or PMA (Fig.1). Although PLD is the major source of stimulus-induced PA formation in permeabilized platelets (Coorssen and Haslam, submitted), the formation of PEt by transphosphatidylation in the

presence of ethanol is a definitive indicator of PLD activity [42]. In the presence of 200 mM-ethanol neither BW755C or indomethacin affected secretion or the formation of [^{3}H]PEt caused by GTP[S] and/or PMA; pretreatment of platelets with 200 μM-ASA [39] also had no effect on Ca^{2+}-independent secretion or PLD activity (not shown). As a whole, these results confirmed the specificity of the inhibitors used, indicated that AA metabolites did not affect PLD activity or the secretion of [^{14}C]5-HT and suggested that PLA_2 activity, as well as that of PLD, correlated with the GTP[S]-induced secretion of [^{14}C]5-HT.

To establish definitively whether PLA_2 activity correlated with the Ca^{2+}-independent secretion of [^{14}C]5-HT, studies with various PLA_2 inhibitors were carried out at pCa >9 (in the presence of 100 μM-BW755C), using GTP[S] and PMA as stimuli to induce a marked formation of both [^{3}H]AA and [^{3}H]PA and near maximal secretion (Table 3). Under these conditions, nordihydroguaiaretic acid (NDGA, 10 μM) [43,44] proved to be the most potent inhibitor of [^{3}H]AA formation (97 ± 6% inhibition; mean ± S.E., n=2), but had no effect on either secretion or the formation of [^{3}H]PA. Aristolochic acid (100 μM) [45] inhibited the formation of [^{3}H]AA by 61 ± 1% (mean ± S.E.; n=2) and also had little effect on secretion or the formation of [^{3}H]PA. The serine esterase inhibitor, phenylmethylsulfonyl fluoride (PMSF, 2 mM) [46] had effects quite similar to those of aristolochic acid (Table 3). Mepacrine (30 - 200 μM), which has been widely used as a PLA_2 inhibitor [47], and has been reported to inhibit the PA-specific PLA_2 of platelets [48], proved to have

substantial nonspecific effects. At 200 μM, mepacrine inhibited the formation of [^{3}H]AA to the same extent as did aristolochic acid but also caused almost 40% inhibition of [^{3}H]PA formation and about 10% inhibition of secretion. Mepacrine also caused the nonspecific release of [^{14}C]5-HT in control samples (15 ± 1%; mean ± S.E., *n*=4). Ethanol (200 mM), which decreases [^{3}H]PA accumulation via the PLD-catalyzed formation of [^{3}H]PEt, not only inhibited both the formation of [^{3}H]PA and [^{14}C]5-HT secretion but also stimulated [^{3}H]AA accumulation by 51 ± 6% (mean ± range; *n*=2). Thus, in these experiments [^{3}H]PA formation (PLD activity) correlated with [^{14}C]5-HT secretion and neither inhibition nor stimulation of [^{3}H]AA accumulation (PLA_2 activity) had any effect on secretion (Table 3). However, at 100 μM, the PLA_2 inhibitor quercetin [43,44] blocked the accumulation of both [^{3}H]AA (70 ± 5%; mean ± range, *n*=2) and [^{3}H]PA (94 ± 2%; mean ± S.E., *n*=3) caused by GTP[S] and PMA, but inhibited the secretion of [^{14}C]5-HT by only 28 ± 4% (mean ± S.E.; *n*=3).

Relationship between secretion and the activities of PLD and PKC

To investigate the apparent dissociation of [^{3}H]PA formation and secretion in the presence of quercetin, the effects of different concentrations of this flavonoid on Ca^{2+}-independent [^{14}C]5-HT secretion, [^{3}H]PA accumulation and phosphorylation of the endogenous PKC substrate, pleckstrin [49], were measured (Fig. 2). The secretion of [^{14}C]5-HT caused by GTP[S] was markedly inhibited by 20 μM-quercetin; a higher concentration had no additional effect (Fig. 2*a*). In contrast, 20 μM-quercetin did not inhibit PMA-induced

secretion and 100 μM-quercetin caused a slight increase in the secretion of [^{14}C]5-HT. With the synergistic combination of GTP[S] and PMA, a significant inhibition of [^{14}C]5-HT secretion was only seen with 100 μM-quercetin. This flavonoid also inhibited the basal formation of [^{3}H]PA found in control samples and had similar effects on [^{3}H]PA formation caused by GTP[S], in the absence or presence of PMA. However, the formation of [^{3}H]PA caused by PMA alone was only inhibited with 100 μM-quercetin. At the latter concentration, this compound inhibited the formation of [^{3}H]PA to below basal values, irrespective of the stimulus used (Fig. 2*b*). To verify that these inhibitions of [^{3}H]PA formation were largely due to inhibition of PLD activity, these experiments were repeated in the presence of 200 mM-ethanol and PLD activity measured as the formation of [^{3}H]PEt (Fig. 3). The results confirmed that quercetin caused a dose-dependent inhibition of PLD activity under all stimulus conditions tested and that this inhibition accounted for the observed inhibitions of [^{3}H]PA formation (Fig. 2*b*), particularly those associated with GTP[S], in the absence or presence of PMA. The phosphorylation of the endogenous PKC substrate pleckstrin also showed a concentration-dependent pattern of inhibition by quercetin (Fig. 2*c*). However, pleckstrin phophorylation caused by PMA alone appeared to be less sensitive to the effects of quercetin than was that caused by GTP[S] or even GTP[S] with PMA. Thus, with 100 μM-quercetin, the effect of PMA was inhibited by only 22 ± 6%, whereas that of GTP[S] was inhibited by 76 ± 1% (means ± range; n=2). These findings suggest that PMA, a potent activator of PKC, can to some extent

block the inhibitory effects of quercetin. Thus, similarly to its effects on secretion caused by GTP[S] with PMA, 100 μM-quercetin only moderately inhibited pleckstrin phosphorylation (25 ± 8%; mean ± S.E., n=3). As a whole, these results are consistent with the view that PLD activation increases PKC activity and secretion in the presence of GTP[S].

DISCUSSION

A previous study from this laboratory [5] established that Ca^{2+} was not essential to the exocytotic mechanism and thus, under essentially Ca^{2+} free conditions (pCa >9), GTP[S] and PMA could act synergistically to induce a near maximal secretory response; Ca^{2+} increases the rate of secretion but does not appear to have a direct role in the mechanism. We have observed strong correlations between dense granule secretion and the formation of PA by PLD in both the absence and presence of Ca^{2+} [7,10], suggesting that PLD has a regulatory function in exocytosis. Although the Ca^{2+} free condition used in these previous studies dissociated PI-PLC activity from secretion, the possible involvement of PLA_2 was never directly examined since all identified forms of this enzyme in human platelets had been reported to be Ca^{2+}-dependent [11-13, 28]. However, exogenous AA has been shown to stimulate Ca^{2+}-independent secretion from permeabilized chromaffin cells [50] as well as the Ca^{2+}-dependent secretion of insulin from intact or permeabilized pancreatic islets [51] and the secretion of both dense and α-granule constituents from intact platelets [24]. The AA-induced secretion

from pancreatic islets was fully dissociated from the formation of eicosanoids [51] but secretion from intact human platelets has appeared to be dependent on the synthesis of AA metabolites [24]. To determine whether the results of this latter work indicated a direct effect of eicosanoids on the exocytotic mechanism, or a potentiating effect on secretion in the presence of Ca^{2+}, the effects of exogenous AA on permeabilized platelets, in both the absence and presence of cyclooxygenase and/or lipoxygenase inhibitors, were investigated in the effective absence of Ca^{2+}. Neither of the compounds used (indomethacin or BW755C) had any effect on the secretion of dense granule constituents. 10 μM-AA potentiated GTP[S]-induced secretion but had no effect alone or in the presence of GTP[S] with PMA. Ten-fold higher concentrations of oleic acid were required for a similar effect although low concentrations (40 μM) of this lipid, which had no effect in either the absence or presence of GTP[S] or PMA, could further enhance the potentiation of secretion caused by the metabolically stable PA analogues, thio-PA and PEt (Coorssen and Haslam, unpublished observations). These results indicated that eicosanoids were not directly involved in the secretory mechanism and that fatty acids, in particular AA, could have a specific effect, perhaps by increasing the PKC activity known to be essential for Ca^{2+}-independent secretion [7]. Higher concentrations of AA (40 - 100 μM) also promoted secretion but this was more likely to be attributed to a detergent-like (lytic) effect, which is known to occur in this concentration range [51].

Thus, the possibility of an involvement of AA itself in

secretion was established. To determine whether the endogenous formation of AA was sufficient to exert a similar effect and whether this formation correlated with the secretion of dense granule constituents, the formation of [^{3}H]AA was measured in permeabilized platelets containing ^{3}H-labelled phospholipids. To ensure that this approach constituted a meaningful determination of PLA_2 activity, which is the major source of free AA in stimulated platelets [52], both indomethacin and BW755C were used to block the rapid metabolism of this fatty acid by the cyclooxygenase and lipoxygenase pathways [12,41,46]. Thus, in both the absence and presence of PMA, secretion induced by GTP[S] correlates with the formation of [^{3}H]AA. A similar correlation between GTP[S]-induced secretion and the formation of [^{3}H]AA were obtained in the presence of Ca^{2+} (pCa 6). These results are consistent with those obtained from measurements of α-granule secretion and [^{3}H]AA formation in intact platelets stimulated with the G protein activator fluoroaluminate, in both the absence and presence of ASA [13]. However, in the present study, secretion in both the absence and presence of Ca^{2+} did not correlate with the total accumulation of [^{3}H]AA obtained in the presence of indomethacin or BW755C, arguing against both a direct fusogenic effect of AA in this system or the involvement of AA metabolites.

In order to determine definitively whether the correlation between [^{14}C]5-HT secretion and the formation of both [^{3}H]AA and [^{3}H]PA [7,10] observed in the presence of GTP[S] at pCa >9 was indicative of a role for PLA_2 and/or PLD in the secretory mechanism, a variety of phospholipase inhibitors were used to further analyze

this possible relationship. BW755C was used to block the metabolism of AA in these studies, and measurements of both [^{3}H]PA and [^{3}H]PEt formation in the absence and presence of ethanol, respectively, showed that BW755C did not affect PLD activity. Use of the documented PLA_2 inhibitors NDGA, aristolochic acid and PMSF [43-46] indicated that moderate to complete inhibition of PLA_2 activity ([^{3}H]AA formation) had no affect on secretion. Each of these compounds is believed to inhibit PLA_2 by a different mechanism and none of them had a major affect on PLD activity; the specificity of both aristolochic acid [45] and PMSF [46] for PLA_2 over PLD has previously been established. Only when an inhibitor blocked PLD activity ([^{3}H]PA formation), as observed with both mepacrine and ethanol, was inhibition of secretion observed. The effect of ethanol is particularly interesting since it inhibited both [^{3}H]PA formation and secretion but caused substantial increases in [^{3}H]AA accumulation. Ethanol has previously been reported to inhibit PLA_2 activity in platelet particulate preparations although no inhibitors of AA metabolism were used in these studies [53]. Another alcohol, *n*-hexanol, has also been reported to stimulate the activity of PLA_2 isolated from human platelet membranes [54]. Mepacrine, which has been widely used as an inhibitor of PLA_2 [47,48], has now been shown to inhibit PLD activity as well. Mepacrine has previously been reported to increase thrombin-stimulated PA formation in intact platelets [46], suggesting a difference in the effect of this compound on intact and permeabilized platelets. However, the results of the present study are consistent with our previous

hypothesis that PLD activity has a role in the regulation of secretion. Thus, the only apparent anomaly in this study involved the effect of the flavonoid, quercetin, which abolished PLD activity and substantially inhibited PLA_2 activity but caused only moderate inhibition of secretion stimulated by GTP[S] with PMA.

More extensive studies of the effects of different concentrations of quercetin were carried out to examine this apparent dissociation of PLD activity and secretion. Measurements of PEt formation in the presence of ethanol confirmed that quercetin caused concentration-dependent inhibitions of the PLD activity induced by GTP[S] and/or PMA. Quercetin also inhibited the basal PA formation that is not associated with secretion [10]; this effect correlated with an inhibition of basal pleckstrin phosphorylation. Activation of PKC by GTP[S] in the absence or presence of PMA was also inhibited by quercetin. The inhibitory effect of quercetin on the phosphorylation of pleckstrin caused by an optimal dose of PMA alone [5] was modest, but consistent with the documented ability of quercetin to inhibit PKC [55]. However, this inhibition of PMA-induced phosphorylation was only observed at the highest dose of quercetin tested (100 μM). These results suggest that quercetin inhibits GTP[S]-induced PKC activation by blocking PLD activity.

The ability of quercetin to inhibit the GTP[S]-stimulated activities of both PLD and PKC, and therefore also Ca^{2+}-independent secretion, is consistent with the hypothesis that PLD has a regulatory role in secretion [5,7,10]. However, PMA is apparently able to maintain sufficient PKC activation, even in the presence of

100 μM-quercetin, to preserve secretion, provided GTP[S] is also present. Since PLD activity was totally abolished by 100 μM-quercetin under these conditions, the results suggest a possible unidentified stimulatory component to the effects of quercetin on secretion. However, since quercetin has previously been shown to inhibit both aggregation and secretion in intact platelets [56], such a stimulatory effect is unexpected. In this regard, it is important to note that quercetin and related flavonoids have been shown to inhibit both tyrosine and serine/threonine protein kinases [55] and receptor-mediated secretion from a wide variety of cell types [57]. Related flavonoids have also been shown to cause structural changes and reduced hydration in model bilayer systems [58]. Should PKC or an unidentified protein kinase have an inhibitory role at some step in the secretory process, inhibition of this enzyme by quercetin could result in a more pronounced secretory response to weaker stimuli. We have obtained evidence indicative of an inhibitory component to the effect of high concentrations of PMA on secretion (Coorssen and Haslam, in preparation); if quercetin inhibited this effect, it would promote secretion. In addition, the ability of quercetin to inhibit receptor-mediated secretion in a variety of cells [57] suggests a possible inhibitory effect on G proteins. Perhaps PMA-induced phosphorylation blocks the inhibitory effects of quercetin at this level, thereby preserving much of the normal secretory response. Furthermore, it is possible that quercetin may directly induce a predisposition for secretion as a result of changes in membrane structure and reduced hydration

barriers. Thus, stimulus conditions that could bypass or overcome the inhibitory properties of this compound could still cause a substantial secretory response. In any case, the multiple effects of this flavonoid make it a less than ideal compound with which to examine the relationship between secretion and the activities of PLA_2 or PLD.

As a whole, the results of the present study establish that although GTP[S] causes a Ca^{2+}-independent activation of PLA_2 in permeabilized platelets, this enzyme activity can be dissociated from the secretion of dense granule constituents. This eliminates any major fusogenic role for fatty acids (AA) or lysophospholipids, or any roles for these products or their metabolites in the exocytotic mechanism of platelets. Thus, PLA_2 is not the target of G_E in platelets, a conclusion that has also been reached regarding the role of PLA_2 in human neutrophils [59] and bovine adrenal chromaffin cells [60]. Our results suggest that in the intact platelet the role of PLA_2 in secretion may be exerted solely through the metabolism of its product, AA, to eicosanoids.

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Fig. 1. Lack of effect of BW755C on the Ca^{2+}-independent formation of [^{3}H]PA

Samples of labelled permeabilized platelets were equilibrated and incubated at pCa >9 with no stimulus (control) or with the indicated combinations of GTP[S] (100 μM) and PMA (100 nM), in either the absence (▨) or the presence (■) of BW755C (100 μM). The formation of [^{3}H]PA was determined; values are means ± S.E. from triplicate incubations.

Fig. 2. Dose effects of quercetin on Ca^{2+}-independent secretion, the formation of [^{3}H]PA and the phosphorylation of pleckstrin

Samples of labelled permeabilized platelets were equilibrated and incubated at pCa >9 with no stimulus (control) or the indicated combinations of GTP[S] (100 μM) and PMA (100 nM), in either the absence of other additions (□) or in the presence of 20 μM (▨) or 100 μM (■) quercetin. The secretion of [^{14}C]5-HT (*a*) and the formation of [^{3}H]PA (*b*) were measured in three identical samples; means ± S.E. are shown. Parallel duplicate incubations were carried out with permeabilized platelet suspension containing [γ-^{32}P]ATP for measurement of the phosphorylation of pleckstrin (*c*); means ± range are shown.

Fig. 3. Dose effects of quercetin on the Ca^{2+}-independent formation of [^{3}H]PEt

Samples of labelled permeabilized platelets were equilibrated and incubated at pCa >9 with 200 mM-ethanol and either no stimulus (control) or the indicated combinations of GTP[S] (100 μM) and PMA (100 nM), in either the absence of other additions (□) or in the presence of 20 μM (▨) or 100 μM (■) quercetin. The formation of [^{3}H]PEt was determined; values are means ± S.E. from triplicate incubations.

Fig. I

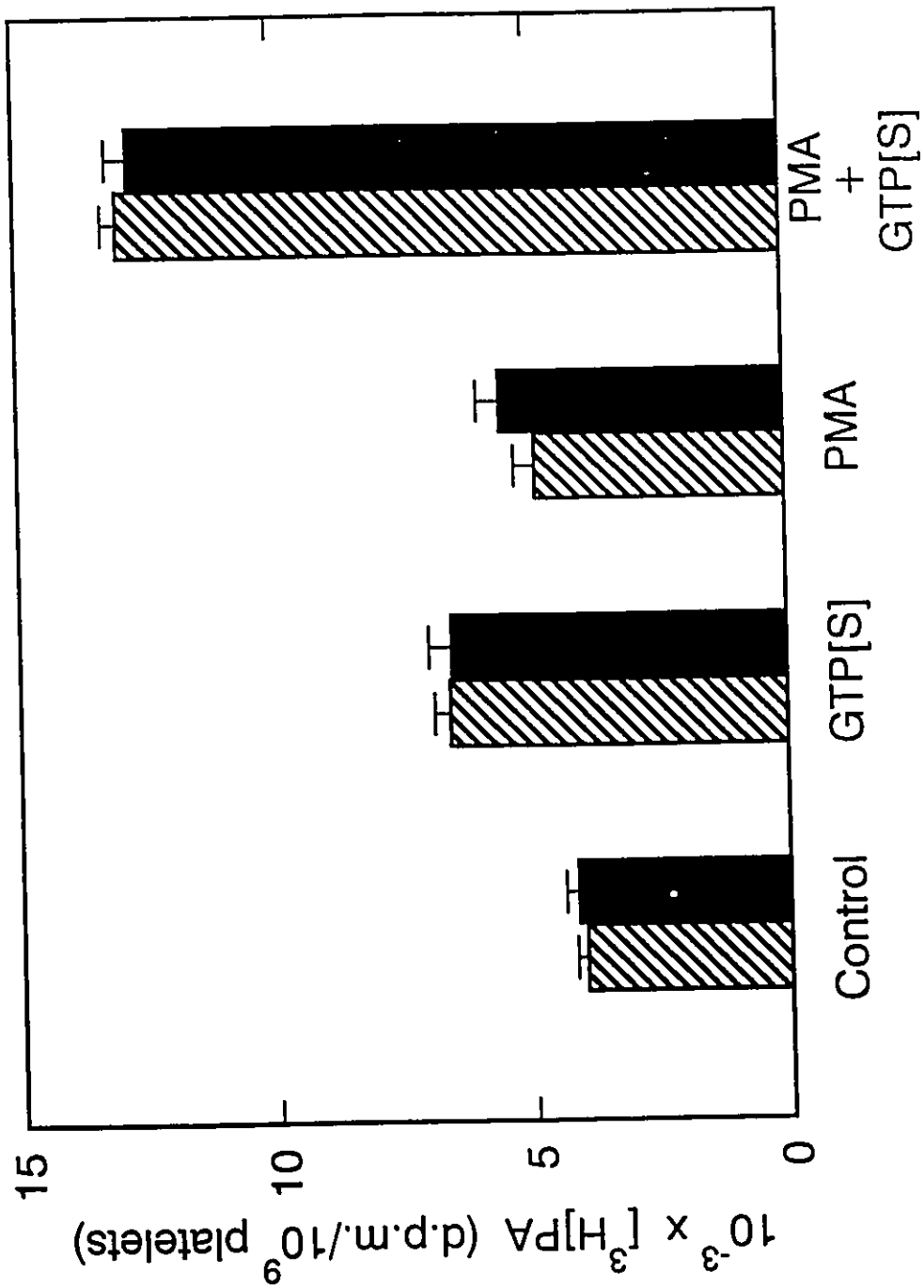

15
10
5
0
10^{-3} x [^{3}H]PA (d.p.m./10^9 platelets)
Control
GTP[S]
PMA
PMA + GTP[S]

Fig. 2

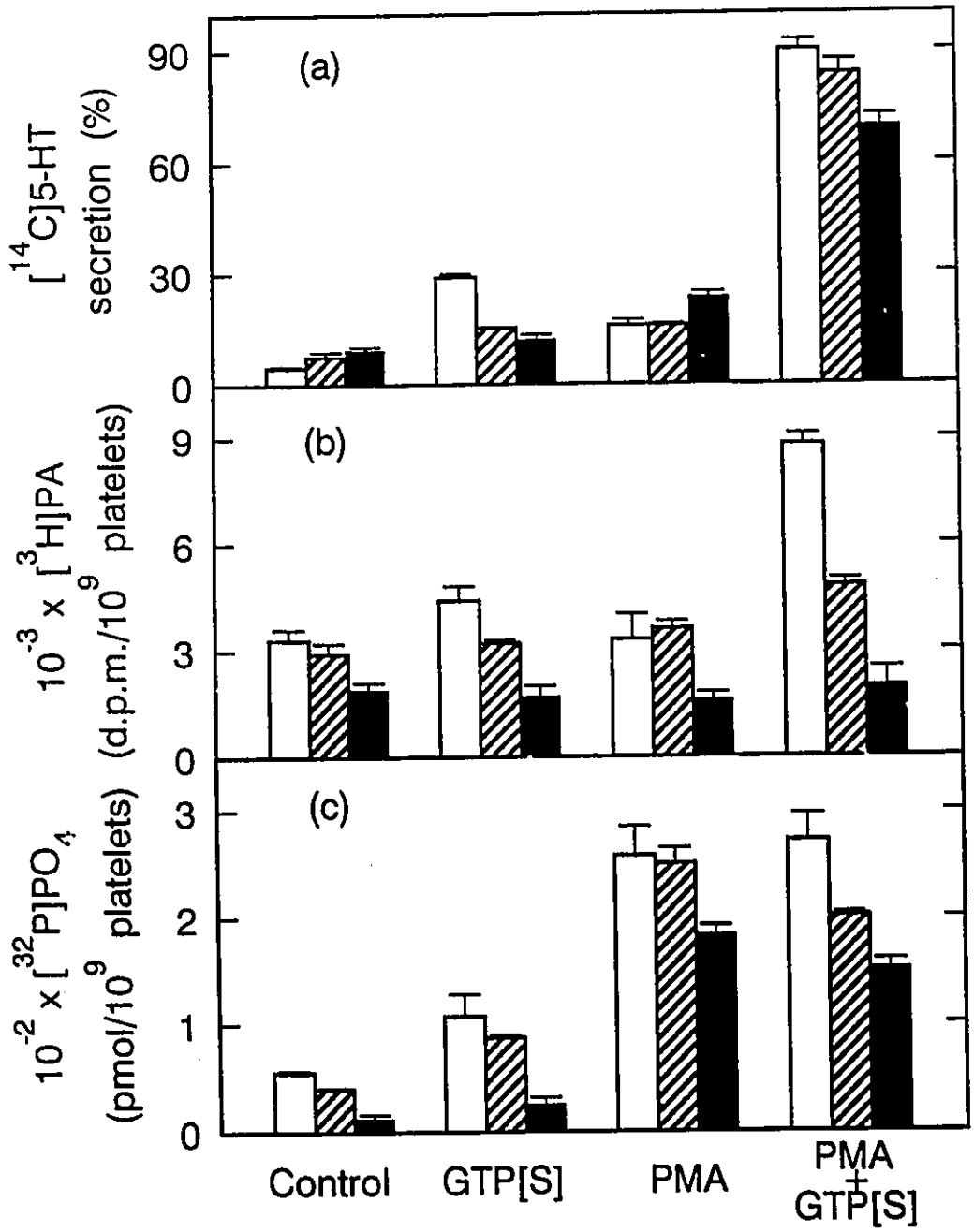

(a)
(b)
(c)
$[^{14}C]$5-HT secretion (%)
$10^{-3} \times [^{3}H]$PA (d.p.m./10^{9} platelets)
$10^{-2} \times [^{32}P]PO_4$ (pmol/10^{9} platelets)
0
30
60
90
3
6
9
1
2
Control
GTP[S]
PMA
PMA + GTP[S]

Fig. 3

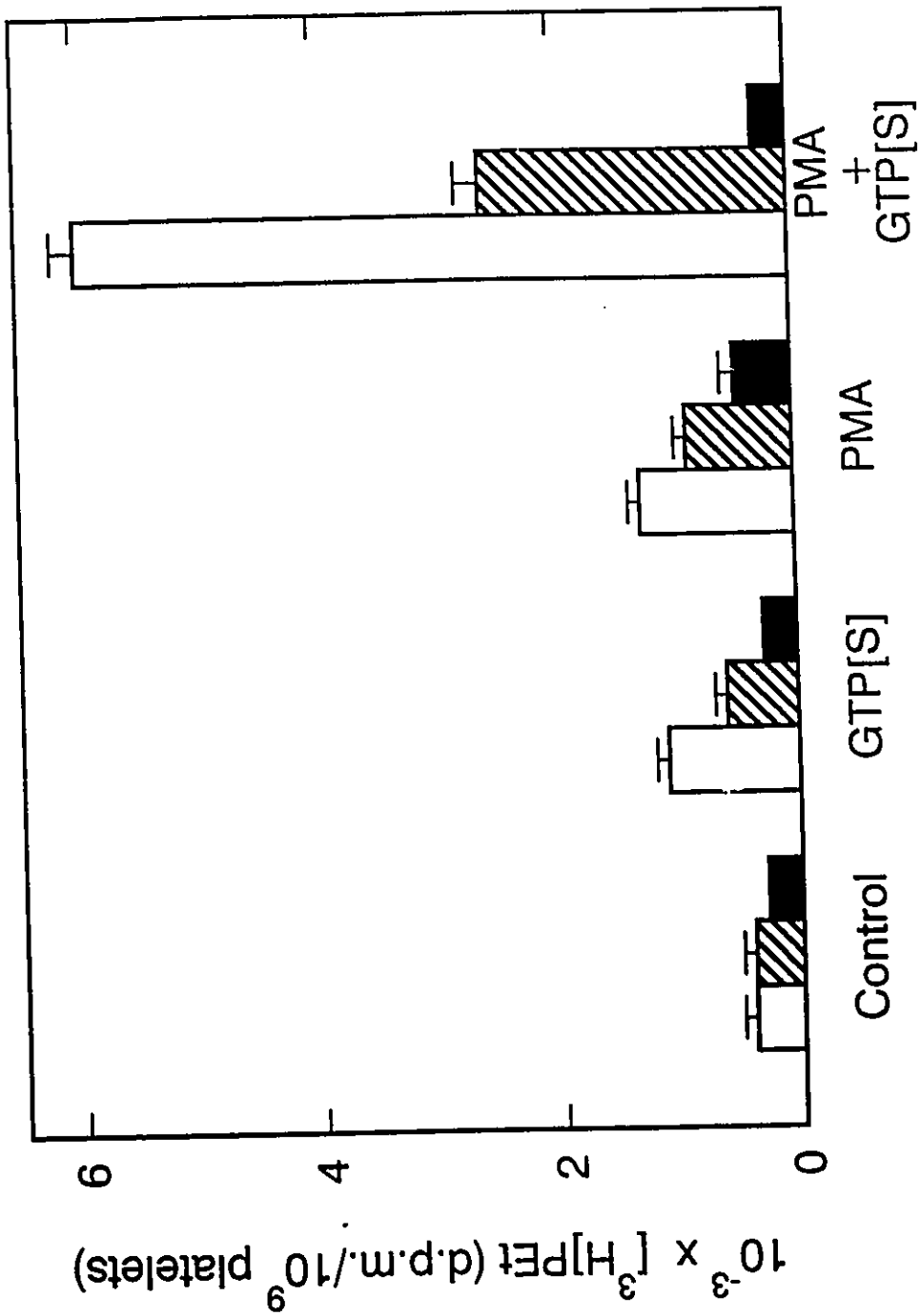


Table 1. Effects of exogenous AA on the Ca^{2+}-independent secretion of [^{14}C]5-HT in the absence and presence of inhibitors of AA metabolism

Samples of permeabilized platelets containing dense granule [^{14}C]5-HT and phospholipids labelled with [^{3}H]AA were equilibrated (15 min at 0°C) and incubated (10 min at 25°C) in the effective absence of Ca^{2+} (pCa >9) [3] with the indicated combinations of GTP[S] (100 μM), PMA (100 nM), indomethacin (20 μM), BW755C (100 μM) and AA. The secretion of [^{14}C]5-HT was determined. Values are means ± S.E. from triplicate samples in the same experiment; significant effects of AA are indicated: * $P < 0.05$; ** $P < 0.01$.

Additions		[^{14}C]5-HT secretion (%) at indicated AA concentrations		
Inhibitor	Stimuli	None	10 μM	40 μM
None	None	4 ± 1	3 ± 1	14 ± 3 *
	GTP[S]	15 ± 0	26 ± 1 **	45 ± 5 **
	PMA	16 ± 0	19 ± 1 *	28 ± 3 *
	GTP[S] + PMA	62 ± 1	64 ± 1	54 ± 8
Indomethacin	None	4 ± 1	4 ± 1	23 ± 4 **
	GTP[S]	13 ± 1	26 ± 4 *	46 ± 4 **
	PMA	15 ± 1	19 ± 1 *	39 ± 4 **
	GTP[S] + PMA	60 ± 2	65 ± 2	77 ± 4
BW755C	None	4 ± 1	6 ± 1	23 ± 3 **
	GTP[S]	15 ± 0	26 ± 1 **	50 ± 3 **
	PMA	16 ± 0	19 ± 0 *	43 ± 0 **
	GTP[S] + PMA	63 ± 1	62 ± 0	82 ± 1 **

Table 2. Effects of GTP[S], PMA and Ca^{2+} on [^{14}C]5-HT secretion and the accumulation of [^{3}H]AA

Samples of labelled permeabilized platelets were equilibrated and incubated in either the absence of Ca^{2+} (pCa >9) or in the presence of Ca^{2+} (pCa 6) with the indicated combinations of GTP[S] (100 μM), PMA (100 nM), indomethacin (20 μM) and BW755C (100 μM). The secretion of [^{14}C]5-HT and the formation of [^{3}H]AA (measured using Solvent 2) were determined in the same samples. The basal concentration of [^{3}H]AA was not subtracted in this experiment. Values are means ± S.E. from triplicate determinations; significant increases in [^{3}H]AA are indicated: * $P < 0.05$; ** $P < 0.01$.

Additions		Secretion of [^{14}C]5-HT	Total of [^{3}H]AA
Inhibitor	Stimuli	(%)	(10^{-3} x d.p.m./10^{9} platelets)
None	None	2 ± 0	4.1 ± 0.3
	GTP[S]	22 ± 1	4.1 ± 0.2
	PMA	12 ± 1	4.1 ± 0.3
	GTP[S]+PMA	59 ± 2	6.3 ± 0.5 *
Indomethacin	None	2 ± 0	6.0 ± 0.3
	GTP[S]	24 ± 1	7.2 ± 0.3 *
	PMA	12 ± 1	5.7 ± 0.3
	GTP[S]+PMA	56 ± 1	8.8 ± 0.2 **
BW755C	None	2 ± 0	5.4 ± 0.2
	GTP[S]	23 ± 3	6.5 ± 0.5
	PMA	12 ± 1	5.2 ± 0.3
	GTP[S]+PMA	58 ± 1	9.3 ± 0.6 **
None	Ca^{2+}	2 ± 1	3.2 ± 0.2
	Ca^{2+}+GTP[S]	53 ± 2	5.7 ± 0.3 **
Indomethacin	Ca^{2+}	2 ± 1	4.4 ± 0.3
	Ca^{2+}+GTP[S]	57 ± 2	7.9 ± 0.3 **
BW755C	Ca^{2+}	3 ± 0	5.7 ± 0.4
	Ca^{2+}+GTP[S]	54 ± 2	12.2 ± 0.5 **

Table 3. Effects of various phospholipase inhibitors on Ca^{2+}-independent secretion and on the associated formation of [^{3}H]AA and [^{3}H]PA

Samples of labelled permeabilized platelets were equilibrated and incubated at pCa >9 with the indicated combinations of GTP[S] (100 μM) + PMA (100 nM) and NDGA (10 μM), aristolochic acid (100 μM), PMSF (2 mM), ethanol (200 mM) or quercetin (100 μM), in each case in the presence of 100 μM-BW755C. The secretion of [^{14}C]5-HT and the formation of [^{3}H]AA (measured using Solvent 1) and [^{3}H]PA were determined in the same samples. Basal values for [^{3}H]AA (0.9 ± 0.1 x 10^3 d.p.m./10^9 platelets) and [^{3}H]PA (0.9 ± 0.1 d.p.m./10^9 platelets) were subtracted; values are means ± S.E. from triplicate incubations.

Additions		Secretion of [^{14}C]5-HT	Formation of [^{3}H]phospholipid metabolites (10^{-3} x d.p.m./10^9 platelets)	
Inhibitor	Stimuli	(%)	[^{3}H]AA	[^{3}H]PA
None	None	2 ± 0	0.4 ± 0.1	0.6 ± 0.1
	GTP[S]+PMA	75 ± 2	2.4 ± 0.1	3.5 ± 0.1
NDGA	None	4 ± 0	0.2 ± 0.2	0.6 ± 0.1
	GTP[S]+PMA	77 ± 2	0.4 ± 0.1	3.4 ± 0.1
Aristolochic acid	None	2 ± 0	-0.1 ± 0.2	0.3 ± 0.2
	GTP[S]+PMA	71 ± 2	0.7 ± 0.2	3.1 ± 0.1
PMSF	None	4 ± 0	0.2 ± 0.1	0.4 ± 0.1
	GTP[S]+PMA	71 ± 1	0.7 ± 0.1	3.0 ± 0.3
Ethanol	None	5 ± 1	0.5 ± 0.2	0.7 ± 0.1
	GTP[S]+PMA	65 ± 2	4.1 ± 0.3	2.1 ± 0.1
Quercetin	None	4 ± 1	0.7 ± 0.2	0.5 ± 0.1
	GTP[S]+PMA	62 ± 2	1.4 ± 0.1	0.7 ± 0.1

GENERAL DISCUSSION

2. Review

The initial studies in this research project (Papers 1 and 2) confirmed that three factors, Ca^{2+}, PKC and a mechanism regulated by GTP-binding proteins, mediate the secretion of both dense and α-granule constituents from electropermeabilized human platelets. No one factor was essential, but any two factors could combine synergistically to yield a pronounced secretory response. Ca^{2+} in particular enhanced the rate and extent of secretion (Paper 1) and PKC may, in some ways, serve a similar function (Paper 3). Studies in permeabilized mast cells have led to the suggestion that Ca^{2+} functions, in part, to enhance the sensitivity of the secretory mechanism to guanine nucleotides and that some PKC-mediated phosphorylation reactions may serve to enhance the sensitivity to both Ca^{2+} and guanine nucleotides (Lillie et al., 1991). Although the patterns of secretion from both dense and α-granules were quite similar under a variety of stimulus conditions (Papers 1 and 2), secretion from the former appeared to be more influenced by PKC activity, whereas secretion from the latter was more sensitive to the G protein-mediated mechanism. Since GTP[S] stimulated Ca^{2+}-independent pleckstrin phosphorylation to only about half the extent seen with an optimal concentration of PMA (and did not enhance the effect of PMA when added simultaneously), but produced substantially more secretion from both dense and α-granules (Papers 1 and 2), the GTP-binding protein involved must stimulate an effector that activates one or more PKC isozymes and/or another protein kinase, as

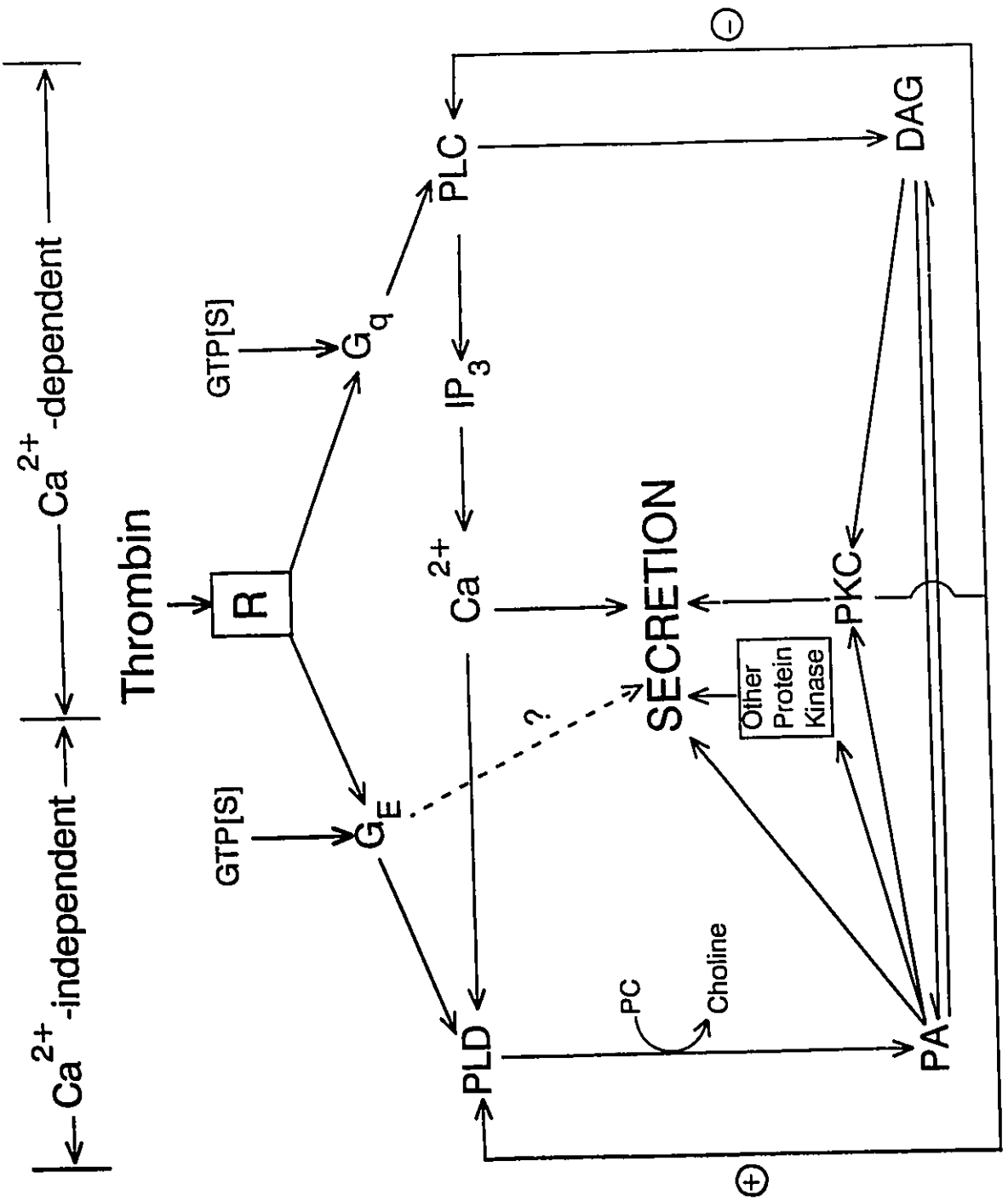
well as having additional effects on the secretory mechanism. The additional effects might even be mediated by another GTP-binding protein-regulated effector. In this regard, the activities of both PLC and PLA_2 were fully dissociated from the exocytotic mechanism (Papers 1-5), which eliminated any direct fusogenic effects of the metabolites of these enzymes, DAG or AA and lysophospholipids, respectively, in the secretory process. Only the activity of PLD was found to parallel closely the secretion of dense granule constituents in both the absence and presence of Ca^{2+} (Papers 3-5). In permeabilized platelets, the combination of GTP[S] and PMA stimulated both Ca^{2+}-independent PLD activity and near-maximal secretion. PLD activity has also been observed in intact platelets stimulated with thrombin concentrations known to induce substantial secretion (Haslam and Coorssen, 1993; Coorssen and Haslam, unpublished observations). Furthermore, in permeabilized platelets, BAPTA and analogues were shown to cause parallel dose-dependent inhibitions of PLD activity and secretion in both the absence and presence of Ca^{2+} (Paper 4), suggesting that these compounds may interact with PLD or its associated G protein(s) and cause inhibition of the enzyme activity by a mechanism unrelated to the ability of these compounds to chelate Ca^{2+}. These findings are likely to explain the previously observed inhibitory effects of BAPTA on Ca^{2+}-independent secretion (reviewed in Knight et al., 1989). Therefore, similarly to the exocytotic mechanism of several other cell types (reviewed in Gomperts, 1990; Tatham and Gomperts, 1991), the secretion of granule constituents from platelets is

controlled by a GTP-binding protein, G_E, and the evidence to date implicates PLD as a possible effector of G_E in platelets (Fig. 1). Similar conclusions have recently been reached regarding the probable role of PLD in neutrophils (Cockcroft, 1992).

2.1. Physiological Significance of PLD Activity

PLD activity correlates with secretion in several cell types including mast cells (Gruchalla et al., 1990; Lin et al., 1991), pancreatic islets (Metz and Dunlop, 1990), neutrophils (Kanaho et al., 1991), adrenal glomerulosa cells (Bollag et al., 1990) and differentiated HL60 cells (Xie et al., 1991; Cockcroft, 1992). PLD activity has also been found to correlate with progesterone production in ovarian granulosa cells (Liscovitch and Amsterdam, 1989), the activation of T lymphocytes (Stewart et al., 1991) and endothelial cells (Martin and Michaelis, 1989; Garcia et al., 1992) and superoxide formation in neutrophils (Bonser et al., 1989). In addition, receptor-linked and/or PKC stimulated PLD activity has also been identified in myocardial cells (Panagia et al., 1991), vascular smooth muscle cells (Konishi et al., 1991), human epithelial HeLa cells (Pepitoni et al., 1991), fibroblast cell lines (Cook and Wakelam, 1992; Kiss, 1992; Wright et al., 1992), human erythroleukemia cells (Wu et al., 1992), Madin-Darby kidney cells (Huang et al., 1992), hepatocytes (Bocckino et al., 1987), astrocytes (Gustavsson and Hansson, 1990), neurons (Chalifa et al., 1990) and various neural-derived cell lines (Lavie and Liscovitch,

Figure 1. Regulation of the exocytotic mechanism as determined from studies on intact and electropermeabilized human platelets. PA is thought to affect secretion through one or more of the following mechanisms: (i) supporting the activation of PKC; (ii) activating an unidentified protein kinase; and (iii) by some unknown, perhaps direct, effect. The possibility that G_E is closer to the exocytotic event, rather than a regulator of PLD, is indicated by the dashed line. "R" represents the thrombin receptor, and all other terms are defined in the text.


Ca2+ -independent
Ca2+ -dependent
Thrombin
R
GTP[S]
GTP[S]
Gq
GE
PLC
IP3
Ca2+
?
SECRETION
Other Protein Kinase
PKC
DAG
PC
Choline
PLD
PA
⊖
⊕

1990; Purkiss et al., 1991; Sandmann and Wurtman, 1991; Holbrook et al., 1992; Horwitz and Ricanati, 1992). Why should PLD activity be found in such a diverse range of cell types and by what mechanism(s) might the enzyme affect the physiological functions that correlate with its activation? The metabolic products of PLD activity must be considered.

Of the PA and phospholipid headgroup moiety released following the activation of PLD, only the lipid has been shown to have possible second messenger functions. Although PC hydrolyzed by PLD has been suggested as a possible source of free choline for the synthesis of acetylcholine in central neurons (Hattori and Kanfer, 1984; Löffelholz, 1989), other functions of free headgroups, particularly in the regulation of secretion seems unlikely, since the addition of various free phospholipid headgroups or phospho-headgroups (products of PLD and PLC, respectively) had no effect on secretion from electropermeabilized platelets (Coorssen and Haslam, unpublished observations). Other possible functions for free headgroup moieties would, from the literature, also appear to be unimportant or at least unexplored. In contrast, PA has been linked to a wide range of cell regulatory functions including the activation of PI-PLC and PLA_2 (Murayama and Ui, 1987; Hashizume et al., 1992), modulatory effects on factors that regulate the activities of low-M_r GTP-binding proteins (Tsai et al., 1989) and the activation of PKC (Ando et al., 1989; Epand et al., 1992; Lee and Bell, 1992) and other protein kinases (Bocckino et al., 1991).

In addition, PA may also promote the Ca^{2+}-dependent binding of annexins to specific membrane sites (Blackwood and Ernst, 1990), or even directly promote membrane destabilization and fusion (Leventis et al., 1986; Park et al., 1992). Recent evidence suggests however, that annexin II has only a modulatory role in the secretory mechanism (Wu and Wagner, 1991) and is therefore unlikely to represent a principal target of PA. Recently, PA has also been identified as a specific activator of PIP kinase from bovine brain (Moritz et al., 1992) and may thus contribute to the maintenance of PIP_2 levels; Eberhard et al. (1990) have previously noted that phosphoinositides are necessary for secretion from chromaffin cells, in some capacity other than as substrates for PLC. Furthermore, since PLD can catalyze a transphosphatidylation reaction, the activity of this enzyme in intact platelets might result in the formation of PA as well as other phospholipid species leading to the formation of membrane sites that confer localized fusion competence to the plasma and/or granule membranes. There is, however, unlikely to be a free source of headgroup moieties in the permeabilized platelet preparations due to the substantial dilution to which such soluble compounds would be subject. Localized changes to membrane structure could also affect protein functions (reviewed by Curtola and Bertoli, 1987; Lenaz, 1987; Shinitzky, 1987; Henis, 1989). Such an effect was suggested to account for the stimulation of Na^{+}-Ca^{2+} exchange observed in cardiac sarcolemmal vesicles treated with PLD (Philipson and Nishimoto, 1984). It has also been shown that vinculin, which may have an important role in the interaction of

cytoskeletal elements with the plasma membrane (Luna and Hitt, 1992), binds preferentially to PS and PA (Niggli et al., 1986) and that the association with these lipids promotes the phosphorylation of this protein by cAMP- and cGMP-dependent kinases and by src kinases, and also enhances its susceptibility to cleavage by proteases (Ito et al., 1983). The formation of localized "fusion-sensitive" sites could also assist in the establishment and stabilization of the fusion pores thought to mediate exocytosis (Almers, 1990; Monck et al., 1990). In addition, a role for PA in membrane fusion events other than exocytosis is suggested by the findings of Santini et al. (1990), who showed that PA, as well as PE and cholesterol, was specifically increased in myoblast membranes during the fusion process. However, it is quite possible that PA does not have a direct role in fusion, but functions solely in a signalling capacity.

Therefore, considering these possible broader influences of PLD activity and the correlations of this enzyme activity with a variety of functions in different cell types, it is possible that PA has another, or more general role, in platelet activation. In this regard, the role of the PI-PLC/DAG kinase pathway as the principal source of PA in intact thrombin-stimulated human platelets (Huang et al., 1991; Haslam and Coorssen, 1993) must be emphasized. The rapid formation of PA in response to a variety of platelet agonists, including thrombin (Lapetina and Cuatrecasas, 1979; Lapetina et al., 1981), collagen (Karniguian et al., 1990), PAF (Shukla and Hanahan,

1984) and possibly ADP (Lloyd et al., 1973), has been known for almost twenty years. With thrombin, this appearance of PA is very early, occurring within 2 - 5 seconds of stimulation and is closely parallelled by the secretion of 5-HT (Lapetina and Cuatrecasas, 1979); the secretion of βTG also parallels PA formation in collagen-stimulated platelets (Karniguian et al., 1990). At 37°C, PA increases rapidly, peaks between 2 - 5 minutes after stimulation and then gradually declines over the course of 15 - 20 minutes (Lapetina et al., 1981; Neufeld and Majerus, 1983). PA derived from DAG has also been suggested to play a role in early platelet responses such as shape change (Lloyd et al., 1973) and Ca^{2+} influx (Imai et al., 1982; Shukla and Hanahan, 1984), in addition to its central role in the resynthesis of phosphatidylinositol. The PA formed during agonist stimulation is likely to arise from at least two different sources (Holmsen et al., 1981), and *de novo* synthesis has been suggested to account for the initial increase observed in thrombin-stimulated rabbit platelets (Vickers et al., 1984). It is therefore not surprising that a fraction of the PA observed in stimulated intact platelets is also attributable to PLD activation (Huang et al., 1991). In intact platelets, this PA produced by PLD may simply be part of the larger pool of PA derived from DAG and therefore may not have a specific function in its own right. In this regard, the observed correlations between PLD activity and secretion in permeabilized platelets may simply be a reflection of the involvement of PA in platelet function and the ability of PLD alone to produce PA under the experimental conditions used. If this

is the case, then the studies in permeabilized platelets still show that PA has an important function, but may overemphasize the involvement of PLD.

Alternately, PA formation may still correlate with secretion even though PLD activity is essentially a vestigial biochemical response that may have only some modulatory affect (via PKC) on the secretory process. In this regard it is noteworthy that electropermeabilized neutrophils lose their phagocytic capacity but that particle binding to the surface can still result in the activation of PLD (Fällman et al., 1992). In this model, PA might affect other components of the platelet activation process or even be delivered to a site of vascular damage, consistent with an established mitogenic role for PA in fibroblasts (Krabak and Hui, 1991; van Corven et al., 1992) and epithelial cells (Imagawa et al., 1989), and thus promote repair. However, this model is inconsistent with the inhibitory effects of BAPTA (Paper 4), unless this compound and its analogues affect G proteins that control both PLD activity and another effector involved in the exocytotic process. In comparison, it is noteworthy that bovine adrenal chromaffin cells apparently contain no PLD activity, although PA formation still occurs via the PLC/DAG kinase pathway (Purkiss et al., 1991), consistent with the metabolic role for PA in cell function. These findings may suggest either that PLD activity is *not* important to the general mechanism of secretion *or* that PLD is an important source of PA in only some cell types (e.g. neutrophils, mast cells,

platelets) in which it *is* important for activation and perhaps, more specifically, for secretion. Thus, "secretion" in Fig. 1 could also represent "activation."

2.2. Investigation of the Role of PLD Activation

The studies comprising this thesis have established that strong correlations exist between the activation of PLD and the secretion of dense granule constituents from human platelets. However, in order to prove or disprove a role for PLD activation (PA formation) in the exocytotic mechanism, or a role in the activation process of platelets, more directed studies are needed. These studies should be designed to investigate not only whether PA formation correlates with secretion (or platelet activation in general), but to establish the possible role(s) of this lipid as a second messenger. Such studies are perhaps best initiated by investigating whether the endogenous formation of PA by exogenous bacterial PLD causes enhanced secretion and/or aggregation. In pancreatic islets, exogenous PLD caused increases in PA that correlated with increased secretion (Metz and Dunlop, 1990b). In intact platelets, exogenous PLD has been shown to cause PA production, PIP_2 breakdown and some protein phosphorylation, consistent with platelet activation, but secretion was not measured (Kroll et al., 1989). However, in a preliminary study with electropermeabilized platelets, exogenous PLD had no effect on secretion in spite of substantial PA formation (Coorssen and Haslam,

unpublished observations). Since this PA could only have been formed in the external leaflet of the platelet plasma membrane, these results may indicate that endogenous PLD activity is localized subcellularly and/or that PA is either not readily converted to DAG (which can flip-flop between monolayers) or not subject to translocation to the inner monolayer of permeabilized platelets at 25°C. Therefore, these experiments with exogenous PLD should be repeated with intact platelets, in the absence and presence of protein kinase inhibitors, and aggregation monitored prior to the determination of secretion; does PA formation correlate best with aggregation, secretion or neither? The concurrent use of staurosporine and other more specific protein kinase (or protein phosphatase) inhibitors may provide indications as to the possible targets of PA.

2.2.1. PA From PLD

Two approaches will be necessary in order to investigate whether the PA formed by PLD in platelets is different, either chemically or in terms of subcellular localization, from the PA formed by PLC. First, exogenous PA should be added to permeabilized platelet preparations to investigate the possible role(s) of this lipid in platelet function. In preliminary studies, it was established that exogenous PA did not affect secretion from permeabilized platelets (Coorssen and Haslam, unpublished observations), perhaps due to its rapid metabolism (Davidson and

Haslam, unpublished observations); these results also support the hypothesis of a specific subcellular localization of PLD or its products. With the aim of circumventing this degradation and thus allowing exogenous PA to reach its postulated internal target sites, the more metabolically stable thio-derivative was synthesized (Bonnel et al., 1982; Orr et al., 1982). However, this thio-PA had no effect alone, and caused only ~10% enhancement of the Ca^{2+}-independent 5-HT secretion caused by GTP[S] or PMA. Thio-PA did, however, interact synergistically with a suboptimal dose of oleate to produce slightly more substantial increases in secretion (Coorssen and Haslam, unpublished observations). All changes were attributable to a potentiation of PKC activity. Since the thio-PA used was the dioleoyl species, the modest effects may reflect the use of PA with these fatty acids, since only minor effects of exogenous dioleoyl-PA have been seen on secretion from bovine parathyroid cells (McGhee and Shoback, 1990). To address these questions, the species of PA formed during the stimulation of permeabilized platelets in the absence, compared to the presence of Ca^{2+}, must be identified. Thio-PA species equivalent to those formed by PLD (in the absence of Ca^{2+}) should be synthesized and added to permeabilized platelets. Control studies involving the addition of PA and thio-PA to intact platelets are also necessary to eliminate any possible extracellular effect of these lipids. Furthermore, the use of PA photoaffinity analogues such as [^{32}P]diazirine-PA (Brunner, 1989; van der Bend et al., 1992a), that bind covalently to neighbouring lipids and amino side chains of integral membrane

proteins upon photolysis, may also prove useful in identifying the site(s) of action of PA in electropermeabilized as well as intact platelets. However, if PLD activity is specifically localized, the problem with all exogenously supplemented lipids will be to get sufficient quantities to the appropriate site(s) of action; high concentrations will likely be needed to see any effects and thus to establish a role for PA. Alternately, permeabilization with detergent or streptolysin-O (see Section 1.1) might eliminate any access problems.

The Ca^{2+}-independent PA-mediated activation of PKC is the first putative function of PA to be identified in this system. Characterization of this PA-sensitive PKC isoform is required. Since human platelets contain the α, β, δ and ζ isoforms of PKC (Crabos et al., 1991, Grabarek et al., 1992; Baldassare et al., 1992), the Ca^{2+}-independent δ and ζ isoforms might be the species in question. However, Epand et al. (1992) have shown that PA can support the binding and activation (with diolein) of the α, β and γ isoforms from rat brain in the absence of Ca^{2+}, and a Ca^{2+}-independent form of PKC-β may be present in rabbit platelets (Pelech et al., 1991). Furthermore, the α and β isoforms of PKC have been shown (with low Ca^{2+}) to induce exocytosis in PKC-depleted pituitary cells (Noar et al., 1989). The use of different phorbol esters, rather than PMA, may prove useful in the identification of the particular PKC isozyme(s) involved (Ryves et al., 1991), as might the depletion of specific PKC isozymes from a cytosolic concentrate

and the subsequent readdition of this cytosol to streptolysin-O or digitonin-permeabilized platelets which have lost their cytosolic components by leakage. The stimulation of such cytosolically-replenished platelets with GTP[S] and/or different phorbol esters should not only help to identify the PA-sensitive PKC isozyme(s), but could also be used to check for any nonspecific effects of PMA and to identify other cytosolic proteins/factors required by the exocytotic mechanism. A similar approach has been used in other secretory cell types to identify proteins essential to Ca^{2+}-dependent exocytosis (Sarafian et al., 1987; Koffer and Gomperts, 1989; Martin and Walent, 1989; Wu and Wagner, 1991; Morgan and Burgoyne, 1992). Alternatively, pure PKC isozymes could be added to platelets permeabilized with digitonin or streptolysin-O, to identify PA-mediated species that promote secretion.

The second approach to characterizing the PA formed by PLD should be an investigation of the subcellular localization of PLD activity in both permeabilized and intact platelets. These studies should be carried-out in the presence of maximal stimuli and high ethanol concentrations in order to generate substantial amounts of the metabolically stable product, PEt. However, secretory fusion must somehow be inhibited in these experiments in order to determine the location(s) of PEt production. Reduced temperature would be the easiest solution to this problem, provided PLD is still active at -18°C; evidence concerning the PLD of rat brain microsomes suggests that this is likely (Chalifour and Kanfer, 1982). Alternatively,

membrane stabilizers such as the impermeant crosslinker adenosine 2',3'-dialdehyde (Ramakrishna and Shinitzky, 1991) or ajoene, which is thought to inhibit secretion from platelets through a physical modification of the hydrophobic core of the plasma membrane (Rendu et al., 1989), might prove effective. A comparison of both approaches would, however, probably be the most useful. In any case, following the stimulation of PLD activity, the preparations can be chilled, fractionated and the accumulation of [^{3}H]PEt in the plasma membrane and different granule fractions determined. This approach should provide information on the locus of PLD activity or an estimate of the distribution of PLD activity between different membrane compartments. It should be noted that the presence of PLD metabolites (PA or PEt) in the plasma membrane would not provide information as to the specific localization of the enzyme. This is particularly important in intact platelets where secretion occurs via fusion of the granules with highly invaginated regions of the plasma membrane, the open canalicular system (White, 1984). Thus, identification of PEt in the plasma membrane fraction could be indicative of PLD activity at sites that are, for all purposes, fusion-competent intracellular regions of the intact platelet.

2.2.2. What is the Identity of G_E?

Regardless of the membrane(s) in which PLD activity is found, it will be necessary to determine whether the enzyme is regulated by heterotrimeric and/or low-M_r GTP-binding proteins. If PLD is the

target of G_E, then the nature of this G protein may contribute to an understanding of the exocytotic mechanism. This line of investigation may also provide a means to dissociate secretion from PLD activity and possibly to prove or disprove a relationship between the enzyme activity and secretion. The simplest approach to this question would involve the use of fluoroaluminates as stimuli; if PLD activity and secretion are both stimulated, then a heterotrimeric G protein is likely to be involved, and the analysis of the apparent involvement of PLD in secretion remains as it is at present using GTP[S] as a stimulus. However, if only PLD activity or secretion is stimulated, then these processes are effectively dissociated on the basis of their regulation by heterotrimeric and low-M_r GTP-binding proteins. However, caution must be exercised in the interpretation of results obtained using fluoroaluminates. Although it is generally accepted that the fluoroaluminates affect only heterotrimeric G proteins (Kahn, 1991), there is evidence that low-M_r GTP-binding proteins can be affected *in vivo* (Wakelam, 1989); furthermore, the fluoroaluminates have been suggested to inhibit a variety of enzymes, particularly those with nucleotide cofactors or that involve the transfer of a phosphate group, including phosphatases (Bigay et al., 1987).

If a heterotrimeric G protein is implicated, then additions of various activated G_α (or $\beta\gamma$) subunits to streptolysin-O or digitonin-permeabilized platelets may prove useful in identifying the G protein involved. Gomperts and colleagues (personal

communication) have interpreted their most recent data as indicating that G_E is a heterotrimeric G protein. However, although this hypothesis is consistent with the data obtained by these authors, it does not take into account the possibility that the product of a G protein-linked effector (e.g. PA) might also directly or indirectly affect a GTP-binding protein to stimulate exocytosis; this possibility would not necessarily be evident from studies in which only GTP[S] was used to activate GTP-binding proteins. In any case, recent work clearly implicates a rab protein in exocytotic fusion in mast cells (Oberhauser et al., 1992), pancreatic acinar cells (Padfield et al., 1992) and adrenal chromaffin cells (Senyshyn et al., 1992). It is also interesting to consider the neutrophil, in which PLD activation has been linked to superoxide production (Bonser et al., 1989; Kanaho et al., 1991); both processes are stimulated by GTP[S] or fluoroaluminates (reviewed in Cockcroft, 1992; English, 1992), and the latter is also stimulated by PA (Bellavite et al., 1988), suggesting a process regulated by heterotrimeric G proteins. However, recent evidence strongly implicates low-M_r GTP-binding proteins (rac) in the activation of superoxide production (Abo et al., 1991; Knaus et al., 1991), suggesting that, *in vivo*, the product of the G protein-linked effector, PLD, may modulate the activity of the low-M_r GTP-binding proteins. A similar process may also operate in platelets, implying that secretion is actually under the control of a low-M_r GTP-binding protein, the activity of which is promoted by the G protein-regulated production of PA. Alternatively, fluoroaluminates, which

have also been shown to stimulate secretion from mast cells (Sorimachi et al., 1988), as well as from all three platelet granule types (Brass et al., 1986; Rendu et al., 1990; Stasi et al., 1992), may also activate the low-M_r GTP-binding proteins (see above). Since a G protein-regulated protein phosphatase has also recently been identified (Pan et al., 1992), the possible involvement of such an enzyme in secretion, as suggested for mast cells (Churcher et al., 1990b), must also be considered. It will be important to establish whether the similarities between secretion from mast cells and platelets are consistent with similar molecular control mechanisms.

Finally, although the majority of the data presented in the studies comprising this thesis suggest that both dense and α-granules are secreted in a similar manner, particularly in the presence of Ca^{2+}, there do appear to be differences in the regulatory mechanisms responsible for secretion from these two granule types (Paper 1). Thus, it will be important to establish whether or not PLD activation is also potentially involved in the regulation of secretion from α-granules, and what differences exist in the mediation of secretion from dense and α-granules. In this regard, it will be important to repeat the studies employing PLA_2 inhibitors, to investigate whether Ca^{2+}-independent and Ca^{2+}-dependent secretion of both dense and α-granule constituents are both unaffected by the inhibition of this enzyme.

2.3. Therapeutic Implications

Although there is no evidence to date that complete and specific inhibition of PLD would have any effect on intact platelets, the evidence presented in this thesis and elsewhere suggests that PLD may be an important regulator of physiological function in platelets, as well as in other cells such as neutrophils and mast cells. The involvement of platelets in pathological conditions such as thrombosis and related cardiovascular diseases (Weiss, 1983) established the therapeutic regulation of platelet activation as a major target of pharmacologic intervention; the involvement of platelets in other pathological conditions (Page, 1988) including cancer (tumor growth) through the release of growth factors, intravascular inflammatory processes through the release of vasoactive and phagocyte-chemotactic compounds and perhaps even in Alzheimers diease, as a circulating source of the amyloid β-protein precursor (Bush et al., 1990; Cole et al., 1990; Van Nostrand et al., 1990) has further broadened interests in the therapeutic regulation of platelet function. In addition, the apparent involvement of PLD in the regulation of a variety of processes in other cells (see above) suggests that this enzyme might prove to be an important target for the treatment of inflammation and perhaps even of disorders involving impaired secretory functions, as seen with certain forms of diabetes (Pickup and Williams, 1991).

Previously, β,β-dimethylglutaric acid was found to inhibit the

neutral PLD activity in rat brain synaptic plasma membranes, suggesting that carboxylic acids may possess intrinsic PLD inhibitory properties (Chalifa et al., 1990). This may in part explain the findings that BAPTA and analogues (Paper 4), as well as high concentrations of indomethacin (1 mM, Coorssen and Haslam, unpublished observations), can inhibit platelet PLD activity. If a derivative of such compounds were prepared, that retained substantial PLD inhibitory properties without other major effects, such a drug might be considered for therapeutic applications or at least as a starting point for the synthesis of a more specific inhibitor; could N-(o-methoxyphenyl)iminodiacetic acid, the half-BAPTA molecule (Haugland, 1992), be the basis for such a drug design? The aminoglycoside antibiotics, including neomycin and streptomycin, have also been reported to inhibit phospholipases (Mingeot-Leclercq et al., 1990) including PLD (Liscovitch et al., 1991), but the mechanism of this inhibition does not appear to involve a direct interaction with the enzyme. Moreover, neomycin has also been suggested to interact directly with G proteins (Aridor and Sagi-Eisenberg, 1990), which could potentially explain this inhibition; interestingly, neomycin (100 μM) was found to inhibit GTP[S]-induced Ca^{2+}-independent secretion slightly in permeabilized platelets (Coorssen and Haslam, unpublished observations). Might BAPTA also function at the G protein level? Whether or not BAPTA interacts directly with PLD will have to be investigated. Furthermore, although ethanol will impair PA formation by PLD, the possible effects of PEt formation in different cell types have not

been investigated, although this metabolite is known to activate PKC *in vitro* (Asaoka et al., 1988). From the data presented in Paper 5, the flavonoid quercetin would also appear to be a potential starting point in a search for PLD inhibitors; perhaps another flavonoid might prove to be more specific for PLD.

Most notable may be the finding that cAMP exerts inhibitory effects on PLD in human neutrophils (Agwu et al., 1991). Since cAMP is a well-documented inhibitor of platelet function (Haslam et al., 1978; Siess, 1989; Tohmatsu et al., 1989; Siess and Lapetina, 1990), the possible effects of elevated cAMP on human platelet PLD activation should be investigated. Such studies, which could easily be carried-out in permeabilized platelets (Knight and Scrutton, 1984), may provide another approach to analyze the apparent relationship between secretion (or activation in general) and PLD activity.

2.4. Closing

Platelets possess several features that make them practical model systems for the study of exocytosis, including (i) the ease of isolating homogeneous preparations; (ii) the absence of a nucleus or notable protein synthesis and therefore probably little or no constitutive fusion; (iii) no notable endocytic capacity; (iv) the presence of three distinct, well defined secretory granule populations with contents for which well established assays are

available, making studies of differentially regulated secretion practical; and (v) several decades of literature concerning the physiology and pharmacology of platelet responses to a variety of agonists and antagonists. Furthermore, considering the importance of understanding the general mechanism(s) of secretion, and the potential physiological and pharmacological importance of PLD activity in platelets, as well as other cells, the results of this thesis show that a concerted effort to determine the role(s) of PLD activity is certainly warranted. In this respect, it is important to realize that the use of permeabilized platelets and effectively Ca^{2+}–free conditions have, for the first time, permitted a detailed analysis of the role of PLD activation in the absence of PLC activity.

Appendix I. Protocol for Preparation and Use of Electropermeabilized Platelets

Blood was collected into acid citrate dextrose (ACD) anticoagulant (Aster and Jandl, 1964) from healthy human donors who claimed to have been drug-free for at least 10 days. This blood was then centrifuged for 15 min at 1000 r.p.m. in a Sorvall RC-3 centrifuge (37°C) using a head with a maximum radius of 9.83 inches. Platelet-rich plasma was removed and centrifuged for 15 min at 2900 r.p.m. in a Sorvall RC-3 (37°C). Using a siliconized glass Pasteur pipette, the resulting platelet pellet was carefully separated from any contaminating red cells by gentle resuspension in plasma, adjusted to 5 x 10^9 platelets/ml and labelled by incubation at 37°C for 1.5 h with 5 μCi [^{3}H]arachidonic acid/ml (60-100 Ci/mmol; Du Pont, Mississauga, Ont., Canada). [^{14}C]5-HT (55 mCi/mmol; Amersham, Oakville, Ont., Canada) was added in three equal amounts during the last 30 min, to a final concentration of 0.25 μCi/ml; when secretion alone was measured, only the latter label was used. The platelets were again centrifuged and the pellet resuspended in 10 ml of Ca^{2+}-free Tyrode's solution (pH 6.5) containing 5 mM Pipes, 0.35% (w/v) bovine serum albumin, 30 μg of apyrase/ml and 50 units of heparin/ml, and incubated for 15 min at 37°C. Following a 10 min cenrifugation at 2400 r.p.m. in a Sorvall RC-3, the platelets were resuspended at 2 x 10^9/ml in Ca^{2+}-free Tyrode's solution (pH 6.5) containing 5 mM Pipes and allowed to equilibrate at room temperature for ~15 min. If the platelets appeared normal (e.g. no clumping),

5 mM EGTA (K^+ salt, pH 6.5) was added and the washed platelets (2 ml portions) were placed in a chamber formed by 10 cm^2 stainless steel electrodes placed 0.2 cm apart. Bubbles were gently cleared from the filled chamber using a fine plastic pipette and each 2 ml portion was subsequently permeabilized by 10 high-volatage electrical discharges from capacitors (4.5 μF) charged at 3.0 kV; the chamber was cooled between sets of 10 discharges so that the temperature of the platelet suspension never rose above 24°C during the permeabilization process. The platelet suspension was immediately placed on ice following permeabilization. The permeabilized platelets were freed from low-M_r solutes by gel filtration (4°C) of 5 ml portions through 20 x 1.5 cm columns (~35 ml) of Sepharose CL-4B (Pharmacia, Que., Canada) that had previously been washed first with 5 ml of 10% BSA (w/v) in buffer A or B (see below) and then with ~150 ml of buffer A or B alone; these washes were required before every experiment. The platelets were eluted from the columns in a medium (pH 7.4) containing the potassium salts of glutamic acid (160 mM), Hepes (20 mM), EGTA (2.5 mM), EDTA (2.5 mM) and either 12.5 mM $MgCl_2$ (buffer A) or 3.9 mM $MgCl_2$ (buffer B). Immediately after elution, the platelet suspension was diluted (to 5 x 10^8 platelets/ml) with the appropriate buffer (A or B) containing 5 mM ATP (Na^+ salt), and these preparations were stored at 0°C until used (within 30 - 60 min).

Sample Preparation and Incubations

For any given experimental sample, the total concentrations of $CaCl_2$ and $MgCl_2$ required to give the desired $[Ca^{2+}_{free}]$ and $[Mg^{2+}_{free}]$ were calculated using a computer program, based on that of Fabiato and Fabiato (1979), which had been rewritten in Fortran and adapted to run on an IBM PC. In addition, this modified program also contained the binding constants for BAPTA and analogues, and allowed rapid calculation of $[Ca^{2+}_{free}]$ and $[Mg^{2+}_{free}]$, based on total additions of $CaCl_2$ and $MgCl_2$. In the experiments described in Papers 1 and 2, the glutamate medium contained 12.5 mM $MgCl_2$ (buffer A) which corresponded to a $[Mg^{2+}_{free}]$ of about 5 - 6 mM (~3.9 mM MgATP) at pCa values in the range of 8 - 5 (Table 1). The addition of 10 mM EGTA to platelets in this medium (12.5 mM EGTA total; pCa >9 based on the criteria described in Paper 1) results in a reduction of the $[Mg^{2+}_{free}]$ to ~2.7 mM, with no significant change in the concentration of MgATP. In subsequent experiments (Papers 3,4,5), the $[Mg^{2+}_{free}]$ for all samples was adjusted to 5 mM; the platelets were suspended in a glutamate-based medium containing 3.9 mM $MgCl_2$ (buffer B) and sufficient high purity $CaCl_2$ (Merck, Darmstadt, Germany) and $MgCl_2$ (BDH, Ont., Canada) were added to achieve the desired pCa values with 5 mM $[Mg^{2+}_{free}]$ (Table 1).

For all experimental conditions used, mock samples containing all required additions except for platelets, were prepared for all combinations of pCa and $[Mg^{2+}_{free}]$ used (particularly in the case of

additions involving the Ca^{2+} buffers, BAPTA and its analogues). The pH of the $CaCl_2$, $MgCl_2$ and any buffer additions were adjusted so that the final pH of all experimental samples was 7.4, whether in buffer A or B. This final pH, for any given experimental condition, was checked and confirmed in at least three separate mock samples. All experimental equilibration and incubation protocols are described in detail in Paper 1 and any changes are noted in the subsequent papers.

Table 1. Total concentrations of $CaCl_2$ and $MgCl_2$ required to give the indicated free concentrations of Ca^{2+} and Mg^{2+} in buffer A or B

pCa	Buffer	Total Ca (mM)	Free Ca^{2+} (nM)	Total Mg (mM)	Free Mg^{2+} (mM)
>9	A	0.1	0.70	12.5	2.7
	B	0.1	0.85	16.7	5.0
7	A	1.2	100	12.5	5.1
	B	1.2	100	12.4	5.0
6	A	2.3	1000	12.5	5.5
	B	2.3	1000	11.9	5.0
5	A	2.9	10000	12.5	5.9
	B	2.9	10000	11.4	5.0

NOTE: The total $[Ca^{2+}]$ at pCa 9 is assumed to be 0.1 mM in the absence of added $CaCl_2$, as discussed in Paper 1. This value may, in practice, be substantially less, giving a pCa >> 9.

Appendix II: Radioimmunoassay Protocol for βTG

Many α-granule constituents are known to bind extensively to the platelet surface (Stenberg et al., 1984; Capitanio et al., 1985), generally in a Ca^{2+}-dependent manner (George and Onofre, 1982). Although some βTG was known to associate with membranes (Stenberg et al., 1984), this binding appeared to be less extensive than that seen with other α-granule constituents, particularly Platelet Factor 4, and βTG was therefore selected as the marker with which to analyze α-granule secretion.

A clinical RIA kit available from Amersham (Oakville, Ont., Canada) was modified to permit 250 assays/kit by reducing the assay volume from 450 to 90 μl. Total platelet βTG was determined in permeabilized platelets lysed by addition of 0.01 vol. of 10% (w/v) Triton X-100 (Biorad, Mississauga, Ont., Canada). During the initial phase of checking the modified RIA procedure, it was found that a low final concentration of Triton X-100 (~0.001%) had to be present in all sample dilutions since this detergent affected the assayable βTG in lysed platelets stored overnight at 4°C. Failure to include the detergent in all sample dilutions resulted in reduced values for total platelet βTG; in an initial experiment, the assayed values of βTG in samples of freeze-thaw lysate stored in phosphate buffer or 0.001 - 0.1% Triton X-100 for 1 - 8 hours (4°C) were 6 ng/ml and 15 ng/ml, respectively. Thus, the detergent effect occurred even at low concentrations of Triton X-100 after short

periods of storage, and was not reversible by dilution. In addition, a final wash of the antigen-antibody complex at the end of the assay was found to reduce variability between samples and improve the reproducibility of the assay.

General Procedure:

1. βTG standards were reconstituted in 250 μl of sterilized or freshly distilled H_2O, half the volume specified in the Amersham protocol. On the day of the assay, 100 μl of each standard was diluted with an equal volume of phosphate buffer containing 50 mM NaH_2PO_4, 2 mM EGTA (pH 7.4) and 0.002% (w/v) Triton X-100. Standards were always assayed in duplicate to establish a standard curve for each experiment. Due to the potential impurities in commercial Triton X-100, only high quality grades of this detergent (e.g. for electrophoresis or protein purification) were used. The ^{125}I βTG and the βTG antiserum were reconstituted as described in the Amersham protocol and did not contain any detergent. Once reconstituted, these reagents could be stored at 4°C for ~2 weeks.

2. Incubations of experimental samples (10 min, 25°C) were terminated with the addition of an equal volume (200 μl) of ice-cold Ca^{2+}- and Mg^{2+}-free stopping buffer (pH 7.4) containing EDTA (35 mM), glutamic acid (80 mM), Hepes (20 mM) and KOH (89 mM), which was followed by immediate centrifugation at 12 000 x *g* for 1 min

(Eppendorf centrifuge) and recovery of the supernatant. A further dilution of experimental supernatants (triplicate samples) was necessary (generally 2 - 200-fold, depending on the original stimulus conditions). These dilutions were made with the phosphate/EGTA buffer described above, contained a final concentration of ~0.001% Triton X-100 and were left on ice for 1 - 1.5 hours, to allow for the detergent effect prior to assay. For the first trial of any given experimental protocol, at least 2 - 3 different dilutions of each supernatant were assayed to obtain βTG values that corresponded to the central section of the standard curve. In addition, control samples containing βTG standard and any agonists or antagonists that had been used in an experiment, at concentrations that would be found in corresponding dilutions of the experimental samples, were also assayed to confirm that these compounds did not interfere with the RIA. No such interference was noted in any of the experiments conducted.

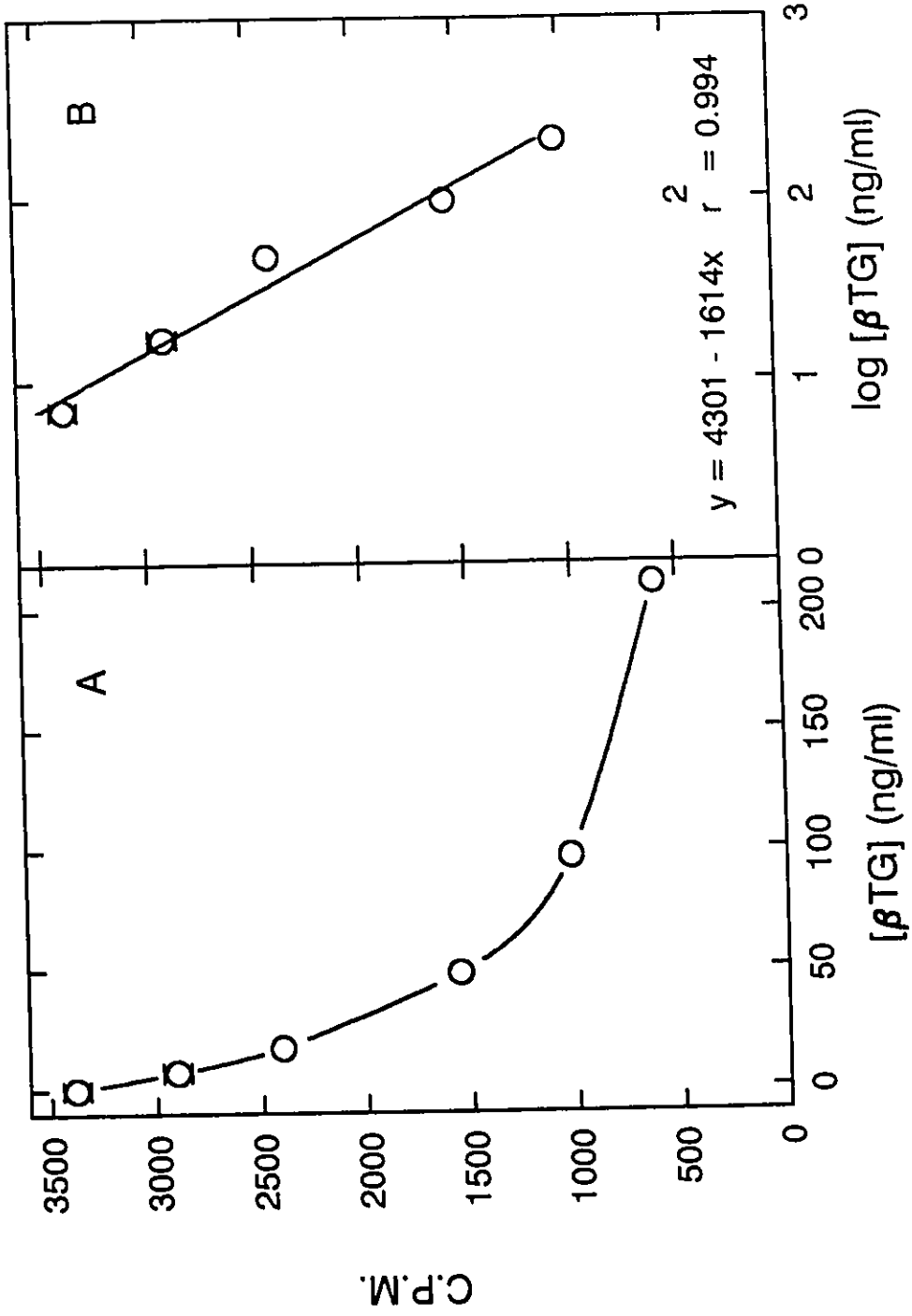
3. After dilution of both βTG standards and experimental supernatants, the assay tubes (0.5 μl Eppendorf tubes) were prepared. Each tube contained 10 μl of either βTG standard, a dilution of experimental supernatant or lysate (all containing ~0.001% Triton X-100), and 40 μl of ^{125}I-labelled βTG. Following the addition of 40 μl of anti-βTG serum (90 μl final assay volume) these samples were vortexed and incubated at room temperature for one hour. The antigen-antibody complex was precipitated with 100 μl of ammonium sulphate (3.3 M) and isolated by centrifugation at 12 000

x *g* for 2 min. The supernatant was removed by vacuum suction. The resulting pellet was washed (without resuspension) with 190 μl of a solution containing phosphate/EGTA buffer with 0.001% Triton X-100 and 3.3 M ammonium sulphate (90 : 100, v/v) and centrifuged as above. The resulting supernatant was again discarded. The bottom of each tube, with the intact pellet, was cut off, placed in a plastic Beckman vial and counted for 10 min in a Beckman Gamma 5500 Counting System.

4. Background counts were subtracted from each c.p.m. value obtained and a standard curve of c.p.m. vs. [βTG] was plotted (Fig. 2A), similar to that described in the original Amersham protocol: the data were verified according to the internal quality control checks described. To facilitate data analysis, the standard curve was linearized by plotting c.p.m. vs. log[βTG] and the line-of-best-fit calculated by linear regression (Fig. 2B). The coefficient of determination (r^2) was always between 0.95 - 1.00. An acceptance zone between -10 - 140 ng/ml βTG was established for data obtained from the 90 μl assay samples, and data not meeting this criterion were rejected. Using the equation for the line-of-best-fit, the RIA data for the experimental samples was then converted to corresponding values of βTG (ng/ml) and this, in turn, was used to calculate the amount of βTG in the original experimental supernatants. These values were then expressed as a percentage of the total platelet βTG determined in the Triton X-100 lysates. Using this method, determinations of the maximum levels of βTG

secreted were similar to those reported by others (Akkerman et al., 1982; Rink et al., 1983; Krishnamurthi et al., 1986).

Figure 2. Standard curve for the assay of βTG obtained using the modified RIA protocol. (A) standard curve of c.p.m. vs. [βTG] as described in the analysis suggested by Amersham. (B) Linearized standard curve obtained by plotting c.p.m. vs. log[βTG], as used for data analysis in the present studies. The equation for the resulting straight line obtained by linear regression appears at the bottom of (B).


C.P.M.
3500
3000
2500
2000
1500
1000
500
0
A
0
50
100
150
200 0
[βTG] (ng/ml)
B
y = 4301 - 1614x
r = 0.994
2
1
2
3
log [βTG] (ng/ml)

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