EFFECTS OF FLUORIDE AND OF VANADATE ON SECRETION FROM ELECTROPERMEABILIZED HUMAN PLATELETS: RELATIONSHIP TO THE ACTIVATION OF PHOSPHOLIPASE D AND PHOSPHOLIPASE C

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ABSTRACT

Platelets permeabilized by high voltage electric discharges have provided a valuable model system in which to analyse the roles of Ca^{2+} ions and guanine nucleotides in the regulation of secretion by exocytosis. In the present study, the effects of fluoride or fluoroaluminate and of vanadate or pervanadate on secretion of platelet dense granule constituents, and the roles of activation of phospholipase D (PLD), phospholipase C (PLC) and protein kinase C (PKC) in secretion, have been investigated. Electropermeabilized human platelets containing [^{14}C]5-HT in their dense granules were suspended in a glutamate medium containing ATP and incubated for 10 min at 25°C with, variously, Ca^{2+} buffers, KF/$AlCl_3$, vanadate/H_2O_2, guanine nucleotides and phorbol 12-myristate 13-acetate (PMA). KF/$AlCl_3$, which activates heterotrimeric G proteins but not low-M_r GTP-binding proteins, caused a Ca^{2+}-dependent [^{14}C]5-HT secretion; maximal effects were obtained with 10 mM KF plus 10 μM $AlCl_3$ at a pCa of 6, when 53% of [^{14}C]5-HT was released. Secretion induced by KF/$AlCl_3$ in the presence of Ca^{2+} correlated with the stimulation of [^{3}H]diacylglycerol accumulation in permeabilized platelets containing [^{3}H]arachidonate-labelled phospholipids. KF/$AlCl_3$ also stimulated the phosphorylation of pleckstrin (P47) in permeabilized platelets incubated with [γ-^{32}P]ATP, indicating the activation of PKC. In the absence of Ca^{2+} (pCa > 9),

$KF/AlCl_3$ caused none of the above effects. These actions of $KF/AlCl_3$ were attributable to the activation of PLC, since $KF/AlCl_3$ also stimulated the formation of [^{3}H]inositol phosphates in [^{3}H]inositol-labelled permeabilized platelets in the presence of Ca^{2+}. PLD activity, measured as the formation of [^{3}H]phosphatidylethanol (PEt) from [^{3}H]arachidonate-labelled phospholipids in the presence of ethanol, could not be detected after stimulation of platelets by $KF/AlCl_3$ in the absence or presence of Ca^{2+}. However, $KF/AlCl_3$ inhibited the [^{3}H]PEt formation (PLD activity) induced by GTPγS. In the absence of Ca^{2+} (pCa >9), the inhibitory effects of $KF/AlCl_3$ on [^{14}C]5-HT secretion induced by GTPγS alone or GTPγS plus PMA correlated well with their inhibitory effects on [^{3}H]PEt formation. At pCa 6, $KF/AlCl_3$ had only a small inhibitory effect on GTPγS-induced secretion and inhibited GTPγS-induced PLD activity more strongly than GTPγS-induced PLC activity. These results suggest that PLD is important for Ca^{2+}-independent secretion, and that, although both PLD and PLC may play roles in Ca^{2+}-dependent secretion, PLC is likely to be the more important. In the presence of Ca^{2+}, either vanadate or H_2O_2 caused concentration-dependent stimulations of [^{14}C]5-HT secretion, [^{3}H]DAG formation and [^{3}H]PEt formation. At pCa 6, low concentrations of vanadate and H_2O_2, which would be expected to form pervanadate, acted synergistically to stimulate [^{14}C]5-HT secretion, which correlated with [^{3}H]DAG formation. However, vanadate with H_2O_2 had a biphasic effect on PLD activity that did not correlate with secretion. In addition, at pCa 6, GTPγS-induced PLD activity was abolished by

vanadate with H_2O_2, whereas GTPγS-induced secretion and PLC activity were only partially inhibited. These results support the idea that both PLC and PLD are involved in the regulation of secretion but have different contributions to Ca^{2+}-dependent and Ca^{2+}-independent secretion. The results are consistent with activation of platelet PLC by a heterotrimeric G protein, but suggest that different mechanisms, possibly involving a low-M_r GTP-binding protein, may be involved in the regulation of PLD activity.

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LIST OF ABBREVIATIONS

AA	arachidonic acid
AlF_4^-	fluoroaluminate
ADP	adenosine 5′-diphosphate
ATP	adenosine 5′-triphosphate
BSA	bovine serum albumin
$[Ca^{2+}_{free}]$	free calcium ion concentration
$[Ca^{2+}_{i}]$	cytoplasmic free calcium ion concentration
cAMP	adenosine cyclic 3′,5′-monophosphate
DAG	*sn*-1,2-diacylglycerol
EDTA	ethylenediamine tetraacetic acid
EGTA	ethyleneglycol-bis-(β-aminoethyl ether) *N,N′*-tetraacetic acid
G protein	guanine nucleotide binding protein
GAP	GTPase activating protein
GDI	GDP dissociation inhibitor
GDP	guanosine 5′-diphosphate
GEF	GDP/GTP exchange factors
GIP	GTPase inhibiting protein
GTP	guanosine 5′-triphosphate

GTPγS	guanosine 5'-*0*-(3-thiotriphosphate)
5-HT	serotonin
HEPES	*N*-2-hydroxyethylpiperazine-*N'*-2-ethanesulphonic acid
ICP-MS	Inductively-Coupled Plasma-Mass Spectrometry
IP	inositol monophosphate
IP_2	inositol 1,4-bisphosphate
IP_3	inositol 1,4,5-trisphosphate
M_r	molecular weight
MLC	myosin light chain
PA	phosphatidic acid
pCa	$-\log[Ca^{2+}{}_{free}]$
PKA	protein kinase A
PKC	protein kinase C
PC	phosphatidylcholine
PE	phosphatidylethanolamine
PEt	phosphatidylethanol
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
PTK	protein tyrosine kinase
PS	phosphatidylserine
PIPES	piperazine-*N,N'*-bis(2-ethanesulphonic acid)
TXA_2	thromboxane A_2
VWF	von Willebrand factor

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1. INTRODUCTION

1.1. Signal transduction in platelets

Blood platelets are important in haemostasis and arterial thrombosis in response to blood vessel damage or exposure of blood to foreign surfaces. The major function of platelets, in which there is no nucleus, is to arrest bleeding and seal the injury site. In addition, platelets are involved in some congenital disorders of haemostasis. The series of platelet responses and interactions during haemostasis and thrombosis are closely linked, i.e. adhesion, shape change, secretion and aggregation (reviewed by Zucker and Nachmias, 1985; Siess, 1989). Collectively these responses constitute platelet activation. Unstimulated platelets are discoid with a smooth surface (Feder *et al.*, 1985). Upon stimulation, the initial responses of platelets are adhesion and shape change. The adhesion of platelets to the subendothelium is mediated by plasma membrane glycoproteins, such as GPIb/IX (receptor for von Willebrand factor [VWF]) (Collier *et al.*, 1983; DeGroot and Sixma, 1990), GPIa-IIa (receptor for collagen) (Nieuwenhuis *et al.*, 1986) and GPIIb-IIIa (receptor for both fibrinogen and VWF) (reviewed by Kieffer and Phillips, 1990). During shape change, platelets lose their discoid shape and become spherical. Pseudopod formation and centralization of granules occur during these processes which involve major rearrangement of the platelet cytoskeleton including the phosphorylation of the

myosin light chain (MLC) (Zucker and Nachmias, 1985). Aggregation requires fibrinogen and Ca^{2+} (Steen and Holmsen, 1987) and is stimulated by the secretion of granule constituents. Platelets have three types of organelle that participate in secretion. Dense granules contain serotonin (5-HT), ADP, ATP and Ca^{2+}; lysosomes contain acid hydrolases; α-granules possess various growth factors and proteins, such as β-thromboglobulin (βTG), fibrinogen, thrombospondin and VWF, which are involved in platelet adhesion or aggregation (Zucker and Nachmias, 1985; Kaplan *et al.*, 1986).

Platelets are very sensitive to a variety of physiological agonists, such as thrombin, collagen fibres, ADP and thromboxane A_2 (TXA_2). In addition, PAF, vasopressin, serotonin, and epinephrine are potent agonists capable of inducing platelet activation (reviewed by Siess, 1989). Secretion of the contents of platelet dense and α-granules can be induced by the above agonists, whereas secretion from lysosomes occurs only upon stimulation by high concentrations of thrombin and collagen (Kaplan *et al.*, 1979). Release of the contents of both dense and α-granules not only triggers the formation of large irreversible aggregates, but also liberates secondary agonists that act on platelets and other cells, including endothelial cells, neutrophils, mast cells, smooth muscle cells, and macrophages (Nozawa *et al.*, 1990). It has also been observed that secretion can occur independently of aggregation, if platelets are stimulated by PAF, vasopressin or TXA_2 and strong platelet-agonists (thrombin, collagen, A23187) (Vargaftig *et al.*, 1981; Krishnamurthi *et al.*, 1984; Siess and Lapetina, 1988; Siess, 1989). Therefore, it is very important to understand the

molecular mechanisms that regulate these secretory responses.

As platelets are one of the most responsive cells to the external signals, the process of platelet activation must be highly regulated. Therefore, the platelet has been widely used as a model for studying signal transduction. The general pathway involved in the transduction of specific extracellular signals from the platelet surface includes a ligand-receptor interaction, followed by the activation of receptor-coupled GTP-binding proteins (G proteins, see section 1.5.) and the subsequent stimulation of phospholipases that generate several types of second messengers by hydrolysing membrane phospholipids. It is well known that the major activation pathway in platelets involves the receptor and G protein-dependent activation of phosphoinositide-specific phospholipase C (PI-PLC), which hydrolyses inositol phospholipids to generate inositol 1,4,5-trisphosphate (IP_3) and *sn*-1,2-diacylglycerol (DAG) as second messengers (Berridge, 1987). IP_3 then triggers the release of Ca^{2+} from intracellular stores; Ca^{2+} may bind to calmodulin, leading to the activation of Ca^{2+}/calmodulin-dependent protein kinases. DAG induces the activation of protein kinase C (PKC) (Nishizuka, 1984; Majerus *et al.*, 1986; Berridge, 1987). Ultimately, these intracellular signals trigger physiological responses through protein phosphorylation, conformational changes in target proteins, or changes in enzyme activities.

It has long been recognized that Ca^{2+} plays an important role in the regulation of exocytosis in platelets as well as in other cells (Winkler, 1988; Knight *et al.*, 1989). The changes in cytoplasmic free Ca^{2+} concentration ($[Ca^{2+}{}_i]$) are

measured by various methods, including the use of $^{45}Ca^{2+}$, the fluorescence indicators, quin 2, fura-2, indo-1 and chlortetracycline or the Ca^{2+}-sensitive photoprotein, aequorin. These studies demonstrated that a large increase in $[Ca^{2+}{}_i]$ in agonist-stimulated platelets resulted from both Ca^{2+} mobilization from one or more intracellular pools and Ca^{2+} influx from outside, via Ca^{2+} channels (reviewed by Haslam 1987; Siess, 1989). Although in resting platelets the $[Ca^{2+}{}_i]$ is 100 nM, there is about several hundred micromolar Ca^{2+} in the dense tubular system (Rink and Sage, 1990). Ca^{2+} ionophores can stimulate secretion from platelets through direct mobilization of Ca^{2+} from intracellular stores. The threshold of $[Ca^{2+}{}_i]$ for Ca^{2+} ionophore (ionomycin)-induced 5-HT secretion is 800 nM (Rink *et al.*, 1982). Vasopressin, PAF and strong platelet-agonists, such as thrombin, collagen and A23187 can stimulate secretion of platelet granule constitutes in the absence of extracellular Ca^{2+} (Siess, 1989). Since the concentration of free Ca^{2+} in plasma (1 mM) is much higher than that in the cytosol of platelets, $[Ca^{2+}{}_i]$ can be increased to 1-2 μM by the opening of a few of Ca^{2+} channels upon stimulation. Indeed, platelet-agonists increased the $[Ca^{2+}{}_i]$ 10-fold more in the presence of external Ca^{2+} than in its absence (Siess, 1989). The actions of both thrombin and ADP involve Ca^{2+} influx from the extracellular space (Sage and Rink, 1986). There is substantial evidence that the increase of $[Ca^{2+}{}_i]$ in platelets can trigger secretion from granule constituents. Activation of protein kinase C (PKC; see section 1.4.) by DAG derived from the activity of PLC enhances the Ca^{2+} sensitivity of secretion (Haslam and Davidson, 1984; reviewed by Siess, 1989; Knight *et al.*, 1989). In thrombin and

collagen stimulated platelets, both a 40-47 kDa protein (P47), which is the major substrate of PKC, and the 20 kDa MLC undergo phosphorylation during platelet activation (Lyons *et al.*, 1975; Haslam and Lynham, 1977; Nishizuka, 1984). MLC is the substrate of Ca^{2+}/calmodulin-dependent MLC-kinase (Daniel *et al.*, 1984) and can also be phosphorylated by PKC at a different site. Phosphorylation of MLC is related to secretion (Painter and Ginsberg, 1984), platelet shape change and contraction (Kroll and Schafer, 1989). However, increasing evidence suggests that secretion can occur independently of the increase of $[Ca^{2+}{}_i]$, as a result of the activation of PKC or the involvement of GTP-binding proteins (Rink *et al.*, 1983; Thompson and Scrutton, 1985; reviewed by Knight *et al.*, 1989; Coorssen and Haslam, 1993).

The activation of platelets involves a complex network of interacting signals generated by the activated receptors, with many positive and negative feedback loops. In addition to the Ca^{2+} and PLC- and PKC-dependent activation pathways, there is substantial support for the involvement of phospholipase D (PLD, see section 1.2.) (Rubin, 1988;. Van der Meulen and Haslam, 1990) and phospholipase A_2 (PLA_2) in the activation of platelets (reviewed by Nozawa *et al.*, 1991). In platelets, PLA_2 hydrolyses phospholipids by cleaving the *sn*-2 acyl bond to release arachidonic acid (AA) and provides lysophospholipid as a substrate for PAF formation. After being released, AA is rapidly converted to biologically active products (eicosanoids) by the cyclooxygenase and lipoxygenase pathways. In human platelets, by the actions of cyclooxygenase and thromboxane synthetase, AA is eventually metabolized to

produce TXA_2, a potent agonist which induces platelet aggregation and secretion of granule constituents. In addition, AA is a well known activator of PLC, PKC and Ca^{2+} mobilization from intracellular storage sites (reviewed by Kroll and Schafer, 1991).

1.2. Phospholipase D

Although increasing evidence suggests that PLD may have a functional role in signal transduction, little is known about the enzyme itself (Exton, 1990). A phosphatidylinositol glycan-specific PLD has been well characterized (Huang, 1990). However, the function of this enzyme in signal transduction is unclear. There are at least two isotypes of mammalian PLD, namely membrane-bound and cytosolic forms. The cytosolic PLD acts on both phosphatidylcholine (PC) and phosphatidylethanolamine (PE), whereas the less abundant membrane-associated PLD specifically hydrolyzes PC (Wang *et al.*, 1991; Huang *et al.*, 1992). PC is the most abundant phospholipid species in mammalian cell plasma membranes and can be hydrolysed by both PLD and PLC (Exton, 1990; Billah and Anthes, 1990). In platelets, the content of PC is higher in intracellular membranes (53%) than in plasma membranes (31%) (Fauvel *et al.*, 1986). PLD hydrolyzes phospholipids (primarily PC) to produce PA which can be further converted to DAG by PA phosphohydrolase. It has been found that PLD exhibited a strict specificity for PC in neutrophils and PC12 cells stimulated by fMet-Leu-Phe and PMA, respectively (Billah and Anthes, 1990; Holbrook *et al.*, 1992). However, PLD was able to catalyze

the hydrolysis of PE or phosphatidylinositol (PI) in several other cell types (reviewed by Billah, 1993).

PLD normally attacks PC, producing free choline and PA through a transient phosphatidyl-PLD intermediate. If the acceptor for the phosphatidyl moiety is water, PA is the sole product; however, if the nucleophilic acceptor is a primary alcohol, the product of this transphosphatidylation reaction is a phosphatidylalcohol. This unique transphosphatidylation reaction catalyzed by PLD provides the basis for a sensitive assay for PLD in intact cells (Billah and Anthes, 1990; Shukla and Halenda, 1991; Thompson *et al.*, 1991). This novel product of phosphatidylalcohol has been used as a powerful tool to distinguish the activation of PLD and PLC, since PC can also be hydrolysed by PLC to produce DAG and phosphocholine, which are interconvertible with PA and choline formed from the activation of PLD.

1.2.1. Regulation of PLD activity. PLD activation can be mediated through multiple mechanisms including interaction with a GTP-binding protein, Ca^{2+} mobilization, activation of PKC and of protein tyrosine kinase (reviewed by Billah and Anthes, 1990).

Studies on intact cells indicate that Ca^{2+} ionophores are potent activators of PLD (Reinhold *et al.*, 1990; Billah *et al.*, 1989a; Huang *et al.*, 1991). In addition, the importance of receptor-mediated Ca^{2+} influx for PLD activation was demonstrated by the observation that addition of a chelator (e.g. EGTA) for extracellular Ca^{2+} in neutrophils blocked the PLD activation by receptor agonists and ionophores (Billah

et al., 1989a; Pai *et al*; 1988). These observations suggest that PLD activation may be caused by an increase in cytoplasmic Ca^{2+} (Billah *et al.*, 1989a; Huang *et al.*, 1991). However, PLD activation occurs in the absence of Ca^{2+} mobilization in PMA-stimulated intact cells (Billah and Anthes, 1990; Shukla and Halenda, 1991; Thompson *et al.*, 1991), as well as in several cell-free preparations including homogenates from endothelial cells, hepatocyte membranes and spermatozoal extracts (Bocckino *et al.*, 1987; Martin, 1988; Domino, *et al.*, 1989). The above evidence suggests the existence of both Ca^{2+}-dependent and Ca^{2+}-independent forms of PLD.

Phorbol ester stimulated PLD activation in rabbit platelets (Van Der Meulen and Haslam, 1990), permeabilized human platelets (Haslam and Coorssen, 1993) and many other cells, such as HeLa cells, neutrophils, HL-60 cells and rat embryonic neurons (reviewed by Shukla and Halenda, 1990). PC hydrolysis was activated by synthetic DAG in rat astrocytes, granulocytes and fibroblasts (Gustavsson and Hansson, 1990; Billah *et al.*, 1989b; Muir and Murray, 1987). PMA-induced activation of PLD can be inhibited by PKC inhibitors (e.g. H7 and K252a) or by prolonged PMA treatment (Liscovitch, 1989; Martinson *et al.*, 1989; Muir and Murray, 1987). These results suggest the involvement of PKC in the regulation of PLD. However, the mechanism by which PKC activates PLD is not identified yet. PMA or PKC may activate PLD by a phosphorylation-independent mechanism (Van Der Meulen and Haslam, 1990; Conricode *et al.*, 1992). Since it is hard to prove that PKC is the only down-stream target of PMA, it is possible that PMA directly acts on PLD. However,

PMA-induced PLD activation was markedly enhanced by overexpression of PKC-β in transfected rat fibroblasts (Pai *et al.*, 1991). Moreover, PLD activities correlated with PKC activation by PIP_2-derived DAG and increasing intracellular Ca^{2+} concentrations, suggesting that PIP_2 hydrolysis is a necessary prerequisite for PLD activation (Billah and Anthes, 1990). These observations indicate a direct function for PKC in the regulation of PLD activation. However, in some cell systems, PLD was activated by receptor-coupled agonists (such as α_2-adrenergic agonists, EGF and α-thrombin) without accompanying PIP_2 breakdown. Apparently, agonist-mediated PLD activation may occur through PKC-dependent mechanisms, as well as those which do not depend on PKC and PIP_2 hydrolysis (reviewed by Billah, 1993).

The regulation of PLD activation by GTP-binding proteins has been established, based on the observations that GTPγS activates PLD in permeabilized cells as well as in cell-free preparations (Van Der Meulen and Haslam, 1990; Harris and Burstein, 1992; Geny and Cockroft, 1992; MacNulty *et al.*, 1992; Coorssen and Haslam, 1993). Although in granulocytes, activation of PLD by receptor-mediated agonists was inhibited by pertussis toxin, which catalyzes an ADP-ribosylation of G_i to block its activation, activation of PLD by receptor-mediated agonists was not inhibited by pertussis toxin in other cells, suggesting the involvement of distinct (pertussis toxin- sensitive and insensitive) G proteins in the regulation of PLD (Agwu *et al.*, 1989; Bocckino *et al.*, 1987; Grillone *et al.*, 1988). The characteristics of this GTPγS-stimulated GTP-binding protein have not been completely identified. It has been found that stimulation of PLD by GTPγS required protein factors in both the

plasma membrane and cytosol in a cell-free system from human neutrophils and granulocytes (Olson *et al.*, 1991; Anthes *et al.*, 1991). Only recently, by using a reconstitution assay, ADP-ribosylation factor (ARF), a low-M_r GTP-binding protein, has been identified as a direct stimulator of PLD in HL60 cells depleted of their cytosol by permeabilization (Brown *et al.*, 1993; Cockcroft *et al.*, 1994). Although Ca^{2+} is required for the stimulation of PLD by GTPγS in neutrophils and HL-60 preparations (Olson *et al.*, 1991; Anthes *et al.*, 1989), GTPγS alone is sufficient for PLD activation in platelet as well as in hepatocyte membranes (Van der Meulen and Haslam, 1990; Bocckino *et al.*, 1987). This observation suggests the existence of an interplay between Ca^{2+}, PKC and GTP-binding proteins in PLD activation.

Evidence is emerging that PLD activation can also be mediated by tyrosine phosphorylation in certain cells. It was found that EGF stimulated PLD activation in Swiss 3T3 fibroblasts without causing IP_3 formation (Cook and Wakelam, 1992). PLD activation was inhibited by protein tyrosine kinase inhibitors (such as ST271, ST638 and erbstatin) but not by PKC inhibitors in human neutrophils (Uings *et al.*, 1992). These results suggest that the PLD activation mediated by tyrosine phosphorylation can be dissociated from the PLC activation.

There is a considerable degree of complexity in the regulation of PLD activities by GTP-binding proteins, Ca^{2+} and PKC. Specific inhibitors which selectively inhibit PLD are necessary to investigate the functional significance of PLD activation further.

1.2.2. Function of PLD in signal transduction. The significance of PLD in control of cell function is uncertain, despite the fact that PA and DAG may be involved in many cellular responses, such as phagocytosis, the respiratory burst, exocytosis and proliferation. PLD may be important in signal transduction not only because it produces PA, which may act on the regulatory proteins for low-M_r GTP-binding proteins, as an activator for PLC, PLA_2 and PKC or as a Ca^{2+} ionophore, but also because it may provide a long term increase in DAG, a well established second messenger, via PA phosphohydrolase (Billah and Anthes, 1990; Nozawa *et al*, 1991).

There is evidence indicating a functional role for PLD-derived PA as a second messenger. Upon stimulation by thrombin in platelets, PA is formed rapidly and is associated with Ca^{2+} influx and release of AA by PLA_2. PA and lyso-PA are found to cause platelet aggregation (Benton *et al.*, 1982). Although exogenous PA could induce Ca^{2+} influx across the plasma membrane in intact cells, the concept that PA acts as a Ca^{2+} ionophore in liposomal or cell systems has been questioned (reviewed by Nozawa *et al.*, 1991). PA is known to be a potent activator of PLC (Jackowski and Rock, 1989), and to be able to replace phosphatidylserine in the activation of PKC (Epand and Stafford, 1990). In addition, PA has been proven to have regulatory effects on low M_r-GTP binding proteins, either by inhibition of ras p21 GTPase activating protein (GAP) (Tsai *et al.*, 1989) or by stimulation of rap1B GTPase inhibiting protein (GIP) (Itoh *et al.*, 1991). Recently, it has been found that PA also stimulates ARF-GAP activity, suggesting that there is a negative feedback in the regulation of PLD (Kahn *et al.*, 1994). Addition of dioleoylthiophosphatidic

acid to platelet supernatant markedly stimulated Ca^{2+}-independent phosphorylation of pleckstrin and MLC (Haslam and Coorssen, 1993). This observation is consistent with the view that PA may stimulate a protein kinase additional to PKC (Bocckino *et al.*, 1991). In addition to stimulating Ca^{2+}-sensitive PKC isozymes, such as PKC-α and PKC-β (Grabarek *et al.*, 1992), PA has been proven to be able to activate Ca^{2+}-insensitive PKC isozymes, including PKC-δ and PKC-ζ (Grabos *et al.*, 1991). Furthermore, addition of PA to fibroblasts stimulates DNA synthesis and cell proliferation, suggesting that PA acts as a mitogenic agent (Yu *et al.*, 1988). The above observations may be sufficient to suggest that PA alone can act as a second messenger.

Many cellular responses initiated by PLD may be due to its contribution to the sustained phase of DAG formation, which could cause long-term activation or down-regulation of protein kinase C (Billah, 1993). There is evidence showing the correlation of PKC activation with DAG derived from PC in other cells (Billah, 1993). The PC-derived DAG may be functionally different from DAG derived from PIP_2 hydrolysis. Leach (1991) has shown that in α-thrombin-stimulated IIC-9 fibroblasts, PKC is not activated by DAG derived from PC, but can be activated by DAG formed from PIP_2 hydrolysis. However, DAG derived from PC can activate PKC from the same cells *in vitro*. It is likely that PC-derived DAG is physically prevented from interacting with PKC, or selectively interacts with PKC isoforms which differ from that activated by PIP_2-derived DAG (Leach *et al.*, 1991). In growth factor-stimulated mitogenesis of IIC-9 cells, DAG and PA are formed by PC

hydrolysis, but not by PIP_2 hydrolysis, suggesting that DAGs derived from PC might have targets other than PKC. However, in most kinds of cells, the functional role of PLD and PA and the products of PA, including DAG, remain to be established.

1.2.3. Function of PLD in secretion. PA itself, rather than DAG, may participate in the coupling of agonist-stimulated secretion in many secretory cells, including platelets, neutrophils, granulosa cells, and mast cells (reviewed by Billah and Anthes, 1990). There is little conversion of PA to DAG in permeabilized platelets (Haslam and Coorssen, 1993). When stimulated with various agonists in platelets, PA formation shows a close correlation with secretion from dense granules (Holmsen *et al.*, 1984). In permeabilized platelets, PLD activities stimulated by GTPγS or PMA correlated well with 5-HT secretion (Coorssen and Haslam, 1993). In other cells, such as chemotactic peptide-stimulated neutrophils and hormone-stimulated granulosa cells, PA formation through PLD correlates with the release of azurophilic granules and aldosterone, respectively (reviewed by Billah and Anthes, 1990). Addition of exogenous PLD to intact granulosa cells induced both aldosterone secretion and PA accumulation (Liscovitch and Amsterdam, 1989). These studies imply the involvement of PA in secretory responses (reviewed by Billah and Anthes, 1990). It was found that PA derived from PLD activation appears to be localized exclusively at the plasma membranes of neutrophils (reviewed by Shukla, 1990). This observation may be related to its function in secretion due to the ability of PA to destabilize model membrane systems, thus initiating membrane fusion (Leventis *et al.*,

1986). In addition, increasing evidence suggests that PLD participates in membrane transport events (reviewed by Liscovitch and Cantley, 1994). However, the mechanism by which PA causes secretion is still unclear.

1.3. Phosphoinositide-specific phospholipase C (PI-PLC)

The functional significance of inositol lipid-specific PLC in signal transduction has been well documented. Although the phosphoinositide content is less than 8% of total phospholipids in mammalian cell membranes, its metabolism plays an important role in signal transduction. In platelets, intracellular membranes are more enriched in phosphatidylinositol (PI) (7%) than is the plasma membrane (3%) (Nozawa, 1991). Polyphosphoinositides (PIP and PIP_2) are found to localize mainly in the plasma membrane. PI can be converted to its 4- and 4,5- phosphorylated derivatives, PIP and PIP_2, via a specific kinase reactions. These three lipids are the predominant phosphoinositides in membranes (Meldrum *et al*, 1991).

PI-PLCs are phosphodiesterases that hydrolyse the glycerophosphate bond of PI, PIP and PIP_2 to generate DAG and the corresponding inositol phosphates, IP, IP_2 and IP_3. In platelets, PLC is activated by a variety of receptor-coupled agonists, such as thrombin, collagen, PAF and TXA_2 (Nozawa *et al.*, 1991). It is well known that the activation of PLC results in the formation of two stimulatory second messengers, IP_3 and DAG. In platelets, the major substrate of PLC is PIP which is hydrolysed into IP_2 and DAG (Culty *et al.*, 1988). Using various purification strategies and molecular cloning technology, the existence of multiple isoforms of PI-

PLC in mammalian tissues has been demonstrated (Rhee and Choi, 1992). There are both soluble and membrane-associated PLCs in platelets, and the activity is present mostly in the cytosolic fraction. There are three main isoforms of PLC, based on sequence homology and deduced amino acid sequences, PLC-β, PLC-γ and PLC-δ. Each possesses a number of subtypes (Rhee and Choi, 1992). Although these isozymes have little sequence homology, two catalytic regions which are named X and Y regions, respectively, are homologous in the three types of PLC (β, γ, δ) (reviewed by Cockcroft and Thomas, 1992). In human platelets, the existence of PLCβ, PLCγ1, PLCγ2 and PLCδ has been reported (Banno *et al.*, 1992). Based on protein purification and biochemical characterization, two more types of PLC (PLCα and PLCε) have been found in human platelets, but these enzymes have not been sequenced or cloned, and their relationship to other PLC isozymes is unclear (Meldrum 1991; Cockroft and Thomas, 1992).

1.3.1. Regulation of PI-PLC activity. There are two distinct pathways for the regulation of PI-PLCs. PLC-β isoforms are regulated by a receptor-coupled G proteins (initially termed Gp), whereas PLC-γ is regulated by receptors with tyrosine kinase activity (Rhee and Choi, 1992). The regulation of other isoforms remain to be determined.

Roles for G proteins in the transduction of signals from agonist-occupied receptors to PLC has been strongly implicated for some time. Evidence has been provided in studies using GTPγS, GTP, AlF_4^- or pertussis toxin with intact or

permeabilized cells or with cell-free membranes (Fain, 1990). By using exogenous substrate combined with protein purification, a G protein present in liver membranes was initially identified to be an activator of PLC (reviewed by Cockcroft and Thomas, 1992). Later, a 42 kDa G protein α subunit belonging to the G_q family, was also identified as the direct activator of PLC. Surprisingly, it has been found that the activation of a single PLC-β needs up to 20 G-protein molecules by *in vitro* reconstitution assay (reviewed by Cockcroft and Thomas, 1992). The G_q family of G proteins, which are capable of activating PLC, has been found to have at least five members, G_q, G_{11}, G_{14}, G_{15} and G_{16} (Simon *et al.*, 1991). The specificity of the interactions between different G_q proteins and PLC-β isoforms has been determined by cDNA transfection assays. For example, it has been found that G_q and G_{11} selectively activate PLC-β1, whereas G_{16} selectively acts on PLC-β2 (Cockcroft and Thomas, 1992). Although the purified α-subunits of G_q can stimulate PLC-β1, it has been found that $\beta\gamma$-subunits could stimulate PLC-β2 purified from HL60 cells, suggesting that $\beta\gamma$-subunits from the G_o and G_i may account for the pertussis toxin-sensitive activation of PI-PLC (reviewed by Sternweis and Smrcka, 1992). Increasing evidence indicates that $G_{\alpha q}$ is responsible for pertussis toxin-insensitive activation of PIP_2 hydrolysis, whereas $\beta\gamma$ subunits of G_i are responsible for the pertussis toxin-sensitive activation of PIP_2 hydrolysis (Liscovitch and Cantley, 1994). This observation may reflect the specificity of G proteins for distinct effectors, which may be important for specific cellular responses. In platelets, there is substantial indirect evidence for a role of G_q in PLC activation (See Section 1.5.). It has been

reported that in TXA_2-stimulated platelets, G_q and G_{11} are involved in the activation of unidentified isoenzymes of PLC (Fain, 1990).

PLC-γ isoenzymes are distinct from other PLC isoenzymes in protein sequence and possess SH2 and SH3 domains similar to those in various tyrosine kinases including $pp60^{src}$. Thus, the EGF-stimulated activation of PLC requires the intrinsic tyrosine kinase activity of the receptor, which appears to be independent of G proteins. It has been found that tyrosine residues of PLC-γ1 can be phosphorylated by growth factor-activated receptors, both *in vivo* and *in vitro*. Immunoprecipitation experiment demonstrated that PLC-γ1 is physically associated with PDGF and EGF receptors through a high affinity interaction between the SH2 domains of PLC-γ1 and the autophosphorylated tyrosine residues of the receptor (reviewed by Rhee, 1991). A correlation between the rapid increase in PIP_2 hydrolysis and tyrosine phosphorylation of PLC-γ1 is observed in EGF-treated cells (Rhee and Choi, 1992). In addition, upon stimulation by EGF or PDGF, an increase in phospholinositide 3-kinase (PI 3-kinase) activity is also observed. PI 3-kinase phosphorylates PI to form PI-3-P, PIP to form PI-3,4-P_2, and PIP_2 to form PI-3,4,5-P_3. Although their functions are still unclear, these 3-phosphorylated phosphoinositides are assumed to be lipid second messengers (Fain, 1990). PI 3-kinase which is activated by a low-M_r GTP-binding protein, Rho, may be involved in the reorganization of the platelet cytoskeleton (Zhang *et al.*, 1993).

It has been found that Ca^{2+} is required for the activities of PLC-β, PLC-γ and PLC-δ. In platelets, the activation of PLC does not require the elevation of

intracellular Ca^{2+} but is dependent on the presence of a basal Ca^{2+} concentration (about 10^{-7} M) (Rhee and Choi, 1992).

The above observations lead to the conclusion that PLCs can be activated by G_q, or growth factors through two distinct pathways. However, PLC can also be inhibited through protein phosphorylation by PKC and PKA. It has been observed that in a variety of cells, addition of PMA and cyclic AMP analogs which are capable of activating PKC and PKA, respectively, results in the inhibition of PLC activation. Possible targets for protein phosphorylation by PKC and PKA include the receptors coupled to G_q or receptor tyrosine kinases and PLC itself. Evidence has been provided that PLCβ1 is phosphorylated by PKC but not by PKA and that serine residues on PLCγ1 can be phosphorylated either by PKC or PKA (Rhee and Choi, 1992). These studies have revealed the existence of a negative feedback regulatory relationship between PLC and PKC, and a regulatory interaction between the PLC pathway and cAMP-dependent pathways.

1.3.2. Function of PI-PLC in signal transduction. Firkin and Williams (1961) first demonstrated phosphoinositide turnover in agonist-stimulated platelets by using ^{32}P-labelled phosphate. Later, it has been determined that the hydrolysis of phosphoinositides by PLC, resulting in the formation of IP_3 and DAG, is an important mechanism for platelet activation (reviewed by Siess, 1989).

It was found that 1 μM IP_3 is able to cause Ca^{2+} mobilization in saponin-permeabilized platelets and in membrane vesicles from smooth endoplasmic

reticulum (Nozawa *et al.*, 1990). The resting platelet has a $[Ca^{2+}{}_{i}]$ of about 100 nM (Rink and Sage, 1990). Upon stimulation by agonists, $[Ca^{2+}{}_{i}]$ can increase to micromolar concentrations. After agonist stimulation, Ca^{2+} ions are immediately released into the cytoplasm, predominantly from a store localized within the dense tubular system (Kroll and Schafer, 1989). IP_3 mobilizes Ca^{2+} from intracellular pools through binding to its receptor which is a Ca^{2+} channel in the endoplasmic reticulum. Recently, receptors for IP_3 have been cloned and sequenced from rat cerebellum (Maeda *et al.*, 1990; Berridge, 1993). Addition of exogenous IP_3 at physiological concentrations to saponin-permeabilized platelets causes Ca^{2+} release from internal stores and this is associated with the platelet responses of shape change, aggregation and secretion (Siess, 1989). The known mechanisms by which Ca^{2+} regulates platelet responses include MLC-kinase (a Ca^{2+}/calmodulin-dependent protein kinase), calpain (a Ca^{2+}-dependent protease) and may also include phospholipases (PLC, PLD and PLA_2) and PKC (Kroll and Schafer, 1989; Ferguson and Hanley, 1991).

IP_3-dependent Ca^{2+} release from intracellular stores can activate Ca^{2+} entry (Berridge, 1993), especially, after internal Ca^{2+} stores have been depleted, and the mechanism for this Ca^{2+} influx may relate to a small unidentified messenger (Randriamampita and Tsien, 1993; Parekh *et al.*, 1993). IP_3 3-kinase, a calmodulin-dependent enzyme, phosphorylates IP_3 to inositol 1,3,4,5-tetrakisphosphate (IP_4) which in some systems may also regulate the influx of extracellular Ca^{2+} in combination with IP_3 (Ferguson and Hanley, 1991; Berridge, 1993). It remains to be determined whether IP_4 itself is a potent intracellular signal in the platelet or only

an inactive form of IP_3 (Ferguson and Hanley, 1991).

The most important function of DAG derived from inositol phospholipid hydrolysis is to activate PKC. Membrane-bound DAG triggers the translocation of PKC from cytosol to the membrane and also increases the affinity of inactive PKC (Ca^{2+}-dependent isozymes) for Ca^{2+}. PKC is then activated by DAG in the presence of Ca^{2+} and phosphatidylserine (Kroll and Schafer, 1989). Addition of chemically modified DAGs to intact platelets causes phosphorylation of pleckstrin (P47), which is a major substrate of PKC (Lapetina *et al.*, 1985a). DAG, in addition to the activation of PKC, can serve a minor source of AA for eicosanoid production (Siess, 1989). DAG may also be involved in the activation of PLA_2 and translocation of DAG kinase to membranes. DAG kinase catalyzes the ATP-dependent conversion of DAG to PA, which is one route for inactivation of DAG (Ferguson and Hanley, 1991). In platelets treated with thrombin or exogenous DAG, addition of inhibitors for DAG kinase enhances the activation of PKC, as well as secretion (Siess, 1989).

Furthermore, DAG is able to promote physicochemical changes in membrane structure, such as an increased membrane curvature or decreased membrane stability (Epand, 1985), and thus influence the activities of phospholipases (Siess, 1989).

1.3.3. Function of PI-PLC in secretion. In thrombin and collagen-stimulated platelets, inositol phospholipid hydrolysis is tightly associated with the secretion from dense granules (Siess, 1989). Thrombin, which induces 70-80% secretion of 5-HT, also induces a higher phospholipase C activation than collagen (Siess *et al.*, 1983).

Secretion can be mediated through Ca^{2+} mobilization and the PKC activation due to the activation of PI-PLC. Studies on permeabilized cells have provided better evidence for this view. Knight and Scrutton (1980) first showed that 50% of 5-HT secretion occurred at about 2 μM [Ca^{2+}_{free}] in the absence of other stimuli. Addition of Ca^{2+} buffers giving pCa values below 6 (>1 μM) also induced the secretion of βTG from α-granules (Coorssen *et al.*, 1990). PMA and thrombin was found to increase the Ca^{2+} sensitivity for the secretion of 5-HT in permeabilized platelets (Knight and Scrutton, 1984; Haslam and Davidson, 1984a). The action of thrombin on secretion was associated with the formation of DAG and the phosphorylation of pleckstrin (Haslam and Davidson, 1984a), indicating a role for PLC, DAG and PKC activation in the secretion. However, there are some examples showing that secretion can occur in the absence of inositol phospholipid hydrolysis. In saponin-permeabilized platelets, in the presence of 1 mM Ca^{2+}, thrombin has been claimed to induce 5-HT secretion without inositol phospholipid hydrolysis and protein phosphorylation (Lapetina *et al.*, 1985b). In addition, GTPβS inhibited DAG formation but not 5-HT secretion stimulated by high concentrations of thrombin (Siess, 1989). Furthermore, activation of PI-PLC is not involved in Ca^{2+}-independent secretion from permeabilized human platelets (Haslam and Coorssen, 1993). These studies imply that secretion from platelets can be mediated by alternative pathways, independently of PI-PLC activation.

1.4. Protein kinase C (PKC)

1.4.1. Isoforms and mechanism of activation. Protein kinase C (PKC) was first identified as a Ca^{2+}-activated, phospholipid-dependent protein kinase in 1977 (Inoue *et al.*, 1977). Since then, studies have shown that PKC consists of a family of serine/threonine kinases with molecular masses ranging from 77-83 kDa. Up to date, 11 isozymes have been identified as the products of specific genes or of alternative splicing of a single transcript. Based on their structures and enzymatic characteristics, PKCs can be divided into four groups. Conventional PKCs include the four PKC isozymes cloned initially, α, βI, βII and γ, which are Ca^{2+}-dependent. Novel PKCs include δ-, ε-, θ- and η-isozymes which are Ca^{2+}-independent (reviewed by Azzi *et al.*, 1992). Atypical PKCs include ζ and ι/λ that are not activated by PMA or DAG. The last group of PKCs includes the recently identified PKC-μ (Dekker and Parker, 1994). Biochemical, immunological and cytochemical studies have shown that expression of PKC isozymes is tissue-specific and differentiation-dependent, suggesting that distinct PKC isozymes may activate different signal pathways (reviewed by Nishizuka, 1988). To date, six isozymes of PKC, α, β, δ, ζ, η' and θ, have been found in human platelets (Crabos *et al.*, 1991; Grabarek *et al.*, 1992; Baldassare *et al.*, 1992; Wang *et al.*, 1993).

Studies on the primary structures show that the conventional group of PKCs contain four conserved regions (termed C_1 to C_4) and five variable regions (V_1 to V_5), whereas the novel group of PKCs lacks the C_2 region responsible for Ca^{2+} binding. The regulatory domain which contains the pseudosubstrate sequence and

interacts with Ca^{2+}, phospholipids, DAG and phorbol ester is located at the amino terminal region containing the C_1, C_2, V_1, V_2 and V_3, whereas the catalytic domain is located at the carboxyl-terminal region containing the C_3, C_4 and V_4 regions (Stabel and Parker, 1991). In platelets, as in most cells, upon activation by DAG, PKC undergoes a translocation from a cytosolic location in resting cells to a membrane-associated site. DAG increases the affinity of PKC for Ca^{2+} so that PKC can be activated by a basal or slightly elevated $[Ca^{2+}{}_i]$ (0.1-1 μM), although under normal stimulated conditions, DAG and elevated $[Ca^{2+}{}_i]$ synergistically stimulate PKC activity (Siess, 1989). During this process, PKC undergoes a conformational change permitting the ready access of substrate to the catalytic site, which is normally blocked by the pseudosubstrate region in the regulatory domain (Stabel and Parker, 1991). PKC can be activated by both an initial increase in the amount of DAG derived from the hydrolysis of phosphoinositides by PLC and a later sustained increase in the amount of DAG derived from the hydrolysis of PC by PLD (Nishizuka 1992). *sn*-1,2-DAGs (Nishizuka, 1984) and phosphatidylserine (PS) are the most effective cofactors supporting PKC activity of the various phospholipids tested so far (Lee and Bell, 1989). Further studies have shown that platelet PKC is activated independently of PLC activation by phorbol esters (Castagna *et al.*, 1982) or synthetic DAG (Lapetina *et al.*, 1985a), because these compounds are cell-permeable and can substitute for endogenous DAG. Phorbol esters are potent PKC activators and have been found to decrease the Ca^{2+} required for the translocation of PKC to membrane-binding sites (Bazzi and Nelsestuen, 1989) or even to activate most PKC

isozymes in the absence of Ca^{2+} (Ryves *et al.*, 1991).

Other mechanisms causing PKC activation include the effects of calpains, Ca^{2+}-activated neutral proteases which cleave membrane-bound PKC to release a fully active 50 kDa enzyme that does not require Ca^{2+}, DAG and PS (Inoue *et al.*, 1977). Calpain I was found to be abundant in human platelets and its activation required micromolar concentrations of Ca^{2+} (Kuboki *et al.*, 1992). Furthermore, it is becoming clear that *cis*-unsaturated fatty acids and lysophosphatidylcholine produced by PLA_2 can activate PKC or enhance its activity (Nishizuka, 1992). In human platelets, PKC could be activated by the simultaneous addition of DAG and *cis*-unsaturated fatty acids, but not saturated or *trans*-unsaturated fatty acids (Yoshida *et al.*, 1992). Collectively, PKC can be activated by DAG and Ca^{2+} from the activation of PLC, and this activation of PKC may be potentiated and prolonged by products derived from the activation of PLD and PLA_2.

1.4.2. Function of PKC in secretion. Studies using phorbol esters or synthetic DAGs demonstrated that platelet PKC activation was associated with aggregation and secretion, but not shape change (Kaibuchi *et al.*, 1983; Kajikawa *et al.*, 1983; Lapetina *et al.*, 1985a). In combination with low concentrations of Ca^{2+} ionophores, phorbol ester or synthetic DAG synergistically stimulated secretion, as well as other responses of platelets, similarly to the effects induced by thrombin (Haslam, 1987; Kroll and Schafer, 1989). It has been observed that thrombin, collagen and ionophore A23187 markedly stimulate the phosphorylation of a 40-47 kDa protein (termed P47) and the

20 kDa MLC (reviewed by Haslam, 1987). P47, now named pleckstrin, identified as the major substrate of PKC in platelets (Nishizuka, 1984), has been purified (Imaoka *et al.*, 1983) and cloned (Tyers *et al.*, 1988). Although the function of pleckstrin has not yet been determined, recently it has been noted that pleckstrin possesses two copies of a domain (PH domain) comprising about 100 amino acids which is present in many proteins involved in signal transduction pathways (Haslam *et al.*, 1993). It has been suggested that PH domains may be involved in interactions with GTP-binding proteins, most likely in binding to the $\beta\gamma$ subunits of G proteins (Musacchio *et al.*, 1993). However, the roles of PH domains are still unclear and are under intense study. Phosphorylation of pleckstrin has served as an index of platelet PKC activation. Nishizuka (1984) has proposed that both PKC activation and Ca^{2+} are required for an optimal platelet response. This concept could account for the secretion from dense granules which was correlated with the phosphorylation of both pleckstrin and MLC (Yamanishi et al., 1983; Haslam et al., 1985), but only partially for the enzyme release from lysosomes (Kajikawa *et al.*, 1983; Knight *et al.*, 1984). Initial studies with electropermeabilized human platelets provided evidence that confirmed this concept (Haslam and Davidson, 1984a). It was found that PMA and thrombin decreased the Ca^{2+} required for 5-HT secretion (Knight and Scrutton, 1984). Addition of PKC inhibitors such as staurosporine or pseudosubstrate peptides inhibited pleckstrin phosphorylation and secretion from dense and α-granules induced by addition of Ca^{2+} buffer alone (Haslam and Coorssen, 1993). These observations indicate that PKC activation is usually involved in the secretion from platelets,

although the mechanism by which PKC causes secretion is unclear. Activated PKC induces the modification of the membrane-bound GPIIb-IIIa complex, so that this complex can bind to fibrinogen, resulting in platelet aggregation. This binding between fibrinogen and GPIIb-IIIa complex can also stimulate signal transduction pathways leading to the secretion (Shattil and Brass, 1987; Banga *et al.*, 1986). In addition, PKC may regulate platelet function by modulating the activation of PLD (Van Der Meulen and Haslam, 1990; Nishizuka, 1992) and PLA_2 (Kroll and Schafer, 1989). Furthermore, PMA inhibited PLC activation suggesting that PKC could exert negative feedback effects on platelet functions (Haslam, 1987; Coorssen *et al.*, 1990).

However, it was found that maximal P47 phosphorylation could occur at concentrations of Ca^{2+} and thrombin which induced little more than a minor secretion (Haslam and Davidson, 1984a). In thrombin and collagen-stimulated platelets, secretion from dense granules was observed without the activation of PLC and pleckstrin phosphorylation (Rittenhouse and Horne, 1984; Lapetina *et al.*, 1985b). These studies suggested that PKC activation may not be absolutely essential to the secretion but rather necessary for potentiation of secretion induced by weak stimuli and that alternative mechanisms rather than the activation of PKC could be involved in agonist-induced secretion.

1.5. GTP-binding proteins

GTP-binding proteins are pivotal in the signal transduction across platelet membranes initiated by diverse stimuli that activate platelets to induce adhesion,

aggregation, granule secretion and the provision of a procoagulant surface that promotes fibrin formation (Brass *et al.*, 1991). Platelets contain at least two major groups of GTP-binding proteins. The first group are heterotrimeric, consisting of three different subunits, α(39-46 kDa), β(37 kDa) and γ(8 kDa) (Boege *et al.*, 1991). The α-subunit, which has GDP/GTP-binding and GTPase activities differs in various G proteins and has been used to define the various heterotrimeric proteins, whereas the attached pair of β and γ subunits can be shared among different α subunits. Relative to α-subunits, β and γ subunit isoforms differ relatively little. There are two interconvertible forms of each G protein, the inactive GDP-bound form of the α subunit which is tightly associated with the $\beta\gamma$ complex and the active GTP-bound form of the α subunit which dissociates from the $\beta\gamma$ complex after GDP/GTP exchange promoted by occupied receptors. Activated α subunits, which possess GTPase activity, hydrolyse the γ-phosphate of the bound GTP. The inactivated α-subunit then reassociates with the $\beta\gamma$ complex. There are at least three functionally distinct G proteins in platelets, 'G_p' which interacts with PLC, and G_s and G_i which interact with adenylyl cyclase (AC) (reviewed by Nozawa *et al.*, 1991). There is as yet no evidence that the G proteins regulating PLA_2 and PLD are heterotrimeric in platelets. Although it is well known that activated α-subunits can transmit signals from the occupied receptor to the effector, recent studies have shown that $\beta\gamma$ dimers can also function as signal transducers (Clapham and Neer, 1993). All the mammalian adenylyl cyclase (AC) gene products (AC I to AC VIII) are activated by α_s, whereas $\beta\gamma$ dimers stimulate AC-II and AC-IV activity, inhibit AC-I activity and

have no effect on the others (Tang and Gilman, 1991). In addition, it has been shown that recombinant PLC-β2 can be activated by βγ dimers at high nanomolar or micromolar concentrations (Camps *et al.*, 1992). It also has been demonstrated that βγ subunits regulate atrial K^+ channels (Neer and Clapham, 1988), the plasma membrane Ca^{2+} pump (Lotersztajn *et al.*, 1992) and PLA_2 (Axelrod *et al.*, 1988).

Studies using nonhydrolyzable analogues of GTP (GTPγS and $G_{pp}(NH)_p$) in permeabilized platelets and platelet membrane preparations, have provided indirect evidence for the involvement of G proteins (termed 'G_p') in the stimulation of PIP_2 hydrolysis (reviewed by Haslam, 1987). It was observed in permeabilized platelets that thrombin and guanine nucleotide analogs (such as GTP, GTPγS and Gpp(NH)p), either alone or in combination, displace the concentration-response curve for Ca^{2+}-induced granule secretion to lower Ca^{2+} concentrations and increase DAG formation, suggesting that PLC is regulated by a G protein in platelets (Haslam and Davidson, 1984a,b,c). Later studies showed that GTPγS or thrombin and GTP stimulated inositol phosphate formation in permeabilized platelets (Culty *et al.*, 1988) or platelet membrane preparations (Hrbolich *et al*, 1987). Studies with other cells, such as neutrophils (Rotrosen *et al.*, 1988), chromaffin cells (Toutant *et al.*, 1987), pancreatic cells (Lambert *et al.*, 1990) and parotid cells (Watson *et al.*, 1992) also demonstrated that heterotrimeric G proteins were present on secretory granules.

The second family of GTP-binding proteins are monomeric and have molecular masses of 20-30 kDa. This family of low-M_r GTP-binding proteins comprises more than 50 members which can be further grouped into several

subfamilies, such as the ras, rab, rho and arf subfamilies, based on their structural differences (Kahn, 1991; Takair *et al.*, 1992). Similarly to heterotrimeric G proteins, low-M_r GTP-binding proteins undergo conformational transitions between inactive GDP-bound and active GTP-bound forms. These two forms can be converted into each other by GDP/GTP exchange or the hydrolysis of GTP, both of which are regulated by various proteins, such as GEF (GDP/GTP exchange factors), GAPs (GTPase-activating proteins) and GIPs (GTPase-inhibiting proteins) (Takai *et al.*, 1992; Macara, 1991).

Early evidence demonstrated that in addition to the heterotrimeric G proteins, numerous low-M_r GTP-binding proteins of 20 to 27 kDa (initially termed G_n) are present in human platelets (Bhullar and Haslam, 1987; Lapetina and Reep, 1987). Some of these have been identified as ral proteins (Bhullar *et al.*, 1990), membranes of the ras subfamily. Recently, the presence of rab3B, rab6 and rab8 on platelet α granules has been demonstrated, suggesting roles for rab proteins in the regulation of secretion (Karniguian *et al.*, 1993). Rho A is known to be present in platelets, but its ADP-ribosylation by botulinum C3 exoenzyme is known to inhibit aggregation rather than secretion (Morii *et al.*, 1992). In addition, rho proteins, may regulate cytoskeletal assembly via the activation of PI 3-kinase (Zhang *et al.*, 1993). Phosphorylation of rap 1B either by PKA or by a Ca^{2+}/calmodulin-dependent protein kinase is thought to decrease the PLC-induced formation of inositol phospholipids; rap 1B forms a complex with both rasGAP and PLCγ in thrombin-stimulated platelets (Farrell *et al.*, 1992). Studies in other cell systems have provided evidence

that low-M_r GTP-binding proteins are involved in secretion. In yeast, products of the YPT1 and SEC4 genes which encode members of the rab subfamily of low-M_r G proteins regulate vesicular transport and constitutive secretion (Kaiser and Schekman, 1990). Studies on the fast axonal transport indicate that low-M_r GTP-binding proteins but not heterotrimeric G proteins (AlF_4^--independent), regulate organelle mobility in a microtubule-based manner (Bloom *et al.*, 1993). ARF isoforms may play important roles in the regulation of both vesicular traffic and PLD activity by serving as a mediator between these two systems (Kahn *et al.*, 1993; Brown *et al.*, 1993). ARF is mainly localized in the Golgi (Stearns *et al.*, 1990), and GTPγS, which stably activates ARF, inhibits the fusion of various vesicles including ER/Golgi, endosome/endosome and nuclear vesicle fusions (Balch *et al.*, 1992; Lenhard *et al.*, 1992; Boman *et al.*, 1992), suggesting an important role for ARF in the regulation of Golgi transport and vesicle fusion in general. In addition, ARF has been found to activate partially purified PLD in a reconstituted assay (Brown *et al.*, 1993; Cockcroft *et al.*, 1994). All the above evidence based on studies on platelets as well as other cells, implies that low-M_r GTP-binding proteins are involved in the regulation of the formation, targeting and fusion of vesicles in the secretory pathway by associating with secretory granule membranes (Pfeffer, 1992; Kahn *et al.*, 1993).

1.6. Factors involved in regulation of secretion from electropermeabilized human platelets

Cell permeabilization has provided a useful means to study cellular signal

transduction mechanisms in many cells. These techniques allow selective breakdown of the plasma membrane, while maintaining the intracellular architecture and their ability to respond to stimuli. Two approaches that are more widely used for platelet permeabilization are electro- and detergent-induced-permeabilization (reviewed by Knight and Scrutton, 1986). Platelets permeabilized by high voltage electric discharges are reasonably stable and apparently homogeneous with minimal leakage of cytosolic components. There is little evidence of plasma membrane damage in electron micrographs, though changes of platelet volume, which are due to cytoskeletal disruption and externalization of plasma membrane invaginations are observed after electropermeabilization (Haslam and Davidson, 1984c; Knight and Scrutton, 1986). Therefore, electropermeabilized platelets have proved particularly useful to study factors involved in the secretion of granule constituents. This approach is biochemically clean. It produces small relatively stable pores of about 2 nm diameter in the plasma membrane, allowing exchanges of small molecules (M_r < 2 kDa) between the cytosol and the extracellular medium (Knight and Scrutton, 1980, 1986). In the method used in this laboratory, compounds released from the platelet cytosol by the electric discharges are removed as completely as possibly by gel filtration of the platelets through Sepharose CL-4B. Storage of the permeabilized platelets (up to 2 hr at 0°C) in a glutamate medium containing MgATP is then possible (Haslam and Davidson, 1984a,b). This approach provides a stable homogeneous preparation of permeabilized platelets without loss of sensitivity to stimuli which cause secretion, though secretion is slower than with intact platelets.

The effects of experimental additions to the platelet preparation can be tested after equilibration with the platelet interior. After warming to 25°C, these platelet suspensions secrete their granule contents through a mechanism involving exocytosis (Haslam and Davidson, 1984a,b; Knight and Scrutton, 1986).

Studies on electropermeabilized human platelets have demonstrated that in addition to the Ca^{2+}-and guanine nucleotide-dependent activation of PLC and PKC (Haslam and Davidson, 1984a, b, c); guanine nucleotides can stimulate secretion of granule constituents by a Ca^{2+}- and PLC-independent mechanism (Coorssen *et al.*, 1990; Haslam and Coorssen, 1993). It has been suggested that an unidentified GTP-binding protein ('G_E') distinct from that involving the activation of PLC, may mediate the PLC and PKC-independent exocytotic process (Gomperts, 1990). Evidence suggesting that phospholipase D (PLD) was one possible target of 'G_E' has been provided by previous studies from this laboratory (Coorssen and Haslam, 1993).

The factors involved in regulation of secretion from electropermeabilized human platelets, are Ca^{2+}, activation of PKC and activation of this unidentified GTP-binding protein ('G_E'). Investigation of the role of PKC in secretion showed that PMA caused increases in the Ca^{2+} sensitivity of both 5-HT (Knight and Scrutton, 1984) and βTG secretion (Coorssen *et al.*, 1990), and stimulated phosphorylation of pleckstrin (Haslam & Davidson, 1984) which is the major PKC substrate and can be used as an indicator of PKC activity (Tyers *et al.*, 1988; Coorssen *et al.*, 1990). The role for a GTP-binding protein in secretion was demonstrated by observations that guanine nucleotides (GTP and GTPγS) also decreased the $[Ca^{2+}{}_{free}]$ required for

both 5-HT and βTG secretion (Haslam and Davidson, 1984b; Coorssen and Haslam, 1990). Further, GTPγS could induce the Ca^{2+}-independent secretion of 5-HT and βTG, and PMA potentiated the effects of GTPγS which were dependent of PKC activity in the absence of Ca^{2+} (Coorssen *et al.*, 1990; Haslam and Coorssen, 1993). Secretion was fully activated in the presence of a combination of any two of three distinct factors, namely Ca^{2+}, PKC activation and activation of a GTP-binding protein. In the presence of Ca^{2+}, either activation of PKC (in the absence of exogenous guanine nucleotide), or addition of GTPγS was sufficient to induce secretion. In the absence of Ca^{2+}, secretion could be induced by GTPγS in combination with PKC activity (Haslam and Coorssen, 1993). Studies in this laboratory have shown a close correlation between PLD activity and Ca^{2+}-independent secretion (Coorssen and Haslam, 1993; Haslam and Coorssen, 1993). GTPγS and PMA acted synergistically to stimulate both secretion and PLD activity, whereas BAPTA could inhibit both secretion and PLD activity by a mechanism that did not involve chelation of Ca^{2+}. PLD activity was measured by the formation of phosphatidic acid (PA) in the absence of ethanol or of phosphatidylethanol (PEt) in the presence of ethanol (Kobayashi and Kanfer, 1987). It was suggested that PA generated by PLD may serve as a second messenger that could mediate secretion from platelets (Haslam and Coorssen, 1993). Although the mechanisms of regulation of secretion from permeabilized human platelets can be simply described in terms of G protein-activated PLC or a G_E-mediated PLD pathway, it is still unclear whether more complex mechanisms that might involve multiple GTP-binding proteins and

target enzymes can be excluded (e.g. various low-M_r GTP-binding proteins and tyrosine protein kinases). Whether the PLD-associated Ca^{2+}-independent secretion was mediated by a low M_r GTP-binding protein, a heterotrimeric G protein or both is still far from clear. It is unknown which type or types of GTP-binding protein are involved in the exocytotic machinery in platelets, because GTPγS is known to activate both monomeric low M_r GTP-binding proteins and heterotrimeric G proteins. In addition, both of these two types of GTP-binding protein have been demonstrated to associate with the membranes of secretory granules (Oberhauser *et al.*, 1992). Furthermore, it is of interest to know whether PLD and PLC have different contributions to Ca^{2+}-dependent- and Ca^{2+}-independent secretion.

1.7. Effects of fluoride in signal transduction

Fluoride (F^-) or fluoroaluminate (AlF_4^-) is a well known G protein activator and has been found to induce cellular activation in many cell types (Sternweis and Gilman, 1982). F^- forms numerous complexes with Al^{3+}, ranging in composition from AlF_1^{2+} to AlF_6^{3-} (Goldstein, 1964). Based on the knowledge of the species of F^- that predominates at the required concentrations of reactants (μM Al^{3+}, mM F^-) (Goldstein, 1964), AlF_4^- was thought to be the active species (Sternweis and Gilman, 1982). Because of the striking structural analogies between AlF_4^- and PO_4^{3-}, AlF_4^- can interact with GDP bound to the α-subunit of transducin to mimic the γ-phosphate of GTP. Thus, the α-subunit of heterotrimeric G protein was proposed to be the target of the AlF_4^- complex, which binds in the nucleotide site close to the

β-phosphate of GDP and induces the switch to a $G_\alpha GDP \cdot AlF_4^-$ form that mimics the active $G_\alpha GTP$ form (Bigay *et al.*, 1985). This model has been confirmed by ^{19}F and ^{31}P NMR spectroscopy of α subunits of G proteins (Higashijima *et al.*, 1991). This study indicated that one GαGDP binds three to five F^- and one single molecule of Al^{3+} and one Mg^{2+} ion. In ^{31}P NMR, binding of F^- and Al^{3+} in the presence of Mg^{2+} caused a change in chemical shift of β-phosphorus in GαGDP to a position similar to that of the β-phosphorus in GαGTP.

Sodium fluoride (NaF) was found to interact with transducin (T) in retinal rods, and with G_i or G_s in other cell systems (Katada *et al.*, 1984). Kahn (1991) observed that F^- was an activator of the heterotrimeric G proteins but was not an activator of low M_r (20-25 KDa) GTP-binding proteins; therefore AlF_4^- can be used to distinguish which type of these two G-protein families is involved in the regulation of cellular activities (Kahn, 1991).

In addition, F^- has been used as an inhibitor of serine and threonine phosphatases (Khandelwal, 1977; Lange *et al.*, 1986). It has been known that F^- inhibits protein phosphatases at millimolar concentrations (Bollen, 1988).

It has been demonstrated that AlF_4^- can either stimulate or inhibit adenylyl cyclase through activation of G_s or G_i (Sternweis and Gilman, 1984). In addition, there is evidence that F^- and AlF_4^- enhance Ca^{2+} sensitivity or induce Ca^{2+} mobilization in many cell types (Kremer *et al.*, 1989; Kawase and Breemen, 1992; Blackmore *et al.*, 1985; Ozaki *et al.*, 1993). In vascular endothelium, fluoroaluminate has been found to stimulate arachidonic acid release through activation of

phospholipase A_2 (Buckley *et al.*, 1991). It has also been reported that NaF stimulates phosphoinositide-specific phospholipase C (PLC) by a mechanism which is dependent upon the activation of a guanine nucleotide binding protein ('G_p') in human neuroblastoma cells (Fisher *et al.*, 1993), but it was also reported that NaF-stimulated phosphoinositide hydrolysis in brain membranes may mediated through a G_p-independent mechanism (Li *et al.*, 1990). NaF-induced inositol phosphate formation has been observed in hamster fibroblasts (Paris and Pouyssegur, 1987), as well as in intact human platelets (Kienast *et al.*, 1987). The activation of PLC by NaF in intact human platelets is independent of ADP, PAF or arachidonate-derived products, but is sensitive to increased levels of intracellular cAMP (Pfliegler *et al.*, 1993; Lazarowski and Lapetina, 1989; Doni *et al.*, 1988). Furthermore, fluoroaluminate has been found to increase PLD activity in permeabilized bovine corneal epithelial cells, as well as in mammalian and avian heart tissue (Akhtar and Choi, 1993; Lindmar and Loffelholz, 1993). These studies suggested that the activation of PLD was modulated by the interaction of a GTP-binding protein, protein kinase C and increased intracellular Ca^{2+}. In neutrophils, F^- activated a Ca^{2+}-independent phospholipase D, which contributed to the accumulation of both DAG and PA (English *et al.*, 1991). It was observed that F^- failed to stimulate PLD activity in the permeabilized NG-108-15 cells (Liscovitch and Eli, 1991), and F^- was also found to inhibit the PA formation stimulated by GTPγS in hepatocyte membranes (Bocckino *et al.*, 1987). These results suggested that F^- may have a direct inhibitory effect on PLD, though the effect of an inhibitory guanine nucleotide-

binding protein cannot be excluded. The different effects of F^- on PLD activity observed in different cell types indicate a relative variability in the phospholipid-dependent signal transduction pathways. The effects of F^- on PLD activity in human platelets have not been investigated.

Studies in intact human platelets have provided evidence that F^--mediated platelet activation is followed by phosphoinositide turnover, increased $[Ca^{2+}{}_i]$, formation of DAG, protein phosphorylation, thromboxane generation, shape change, dense granule release and aggregation (Nakmura *et al.*, 1988; Pfliegler *et al.*, 1993). F^--induced platelet activation was attributed to the activation of a G protein-regulated PLC. However, a conflicting observation suggested that F^- did not stimulate a G protein governing phosphoinositide-specific phospholipase C, but induced platelet activation directly through DAG formation and protein kinase C activation (Rendu *et al.*, 1990). Such contradictory observations have led us to analyze further the mechanism of F^--induced platelet activation in which PLC or PLD might be involved.

1.8. *Effects of vanadate/H_2O_2 in signal transduction*

Vanadate, another phosphate analog, is often used as sodium orthovanadate (Na_3VO_4), though vanadium may exist in several oxidized forms in solution. These are the +4 oxidation state (HVO_2^+) or +5 oxidation state, which includes HVO_4^{2-}, $H_2VO_4^-$ and VO_3^- (Gordon, 1991). Vanadate has been found to stimulate adenylate cyclase activity in turkey erythrocyte membranes via the activation of G_s, similarly to fluoride; however, the stimulatory mechanism was different (Krawietz *et al.*, 1982).

It has also been reported that vanadate and NaF/$AlCl_3$ induced the formation of inositol phosphates by activating PLC in resting hamster fibroblasts (Paris and Pouyssegur, 1987). Vanadate is a well-known inhibitor of protein-tyrosine phosphatases and has been widely used to evaluate the role of protein-tyrosine phosphorylation in various cellular processes (Swarup *et al.*, 1982). When used alone to induce cellular activation, vanadate must be used in high concentrations, incubated for long times, or used with electropermeabilized cells. However, pervanadate {$[VO_2(O_2)_2]^{3-}$} derived by the oxidization of orthovanadate (Na_3VO_4) by hydrogen peroxide (H_2O_2) can enter easily into intact cells (Inazu *et al.*, 1990). Both vanadate and H_2O_2 are known to have physiological effects similar to insulin, based on their activation of the insulin receptor kinase in rat adipocytes (Fantus *et al*, 1989). Recent findings have demonstrated that vanadate with H_2O_2 stimulated protein-tyrosine phosphorylation and aggregation in intact human platelets (Inazu *et al.*, 1990). Furthermore, in electropermeabilized human platelets, it was found that vanadate with molybdate promoted 5-HT and PDGF secretion, as well as increased tyrosine phosphorylation of a 50-kDa protein (Lerea *et al.*, 1989). Both studies suggested that tyrosine phosphorylation of certain proteins might be involved in platelet activation. These data suggested that tyrosine phosphorylation might be part of the platelet activation cascade. A recent report indicated that human platelets could be activated by pervanadate through a mechanism involving tyrosine phosphorylation of PLCγ and the accumulation of inositol phosphates (Blake *et al.*, 1993). Furthermore, pervanadate has been reported to stimulate a PLD activity that is closely associated

with enhanced tyrosine-phosphorylation of certain proteins in electropermeabilized HL-60 cells (Bourgoin and Grinstein, 1992) and in phagocytic leucocytes (Dubyak *et al.*, 1993). These groups had different opinions on whether a G protein-based stimulatory mechanism is involved in the activation of PLD or not. The effects of vanadate/H_2O_2 on 5-HT secretion and the relationship with the activation of PLD and PLC are still unclear in human platelets. It remains to be determined whether platelet activation by vanadate is through a mechanism involving the G protein-mediated activation of PLC or PLD, both of which may also be associated with tyrosine phosphorylation.

1.9. Objectives of this thesis

The current study presents an attempt to analyze the roles of PLD and PLC in the regulation of secretion from electropermeabilized human platelets using KF/$AlCl_3$ and vanadate/H_2O_2 as tools. These agents were chosen because of their potential ability to interact with and activate a variety of intracellular signalling pathways that might have effects on secretion as well as on PLD or PLC. In addition, I have tried to clarify the nature of the GTP-binding proteins (heterotrimeric G proteins or low M_r GTP-binding proteins) that mediate the activation of the PLD involved in signal transduction in platelets, since AlF_4^- does not activate low M_r GTP-binding proteins (Kahn, 1991). The results throw light on the Ca^{2+}-independent secretion mediated by PLD, as well as on the Ca^{2+}-dependent secretion mediated by PLC in permeabilized human platelets.

2. EXPERIMENTAL

2.1. Materials

[*side-chain*-2-^{14}C]5-HT (55 mCi/mmol) and aqueous counting scintillant (ACS) were obtained from Amersham (Oakville, Ont., Canada). [γ-^{32}P]ATP (3000 Ci/mmol) and [5,6,8,9,11,12,14,15-^{3}H]arachidonic acid (100 Ci/mmol) were from Du Pont (Mississauga, Ont., Canada). Myo-[2-^{3}H]inositol (15 Ci/mmol) was obtained from American Radiolabelled Chemicals (St Louis, MO, USA). [^{14}C]DAG was from Amersham (Oakville, Ont., Canada).

GTPγS was from Boehringer Mannheim Canada Ltd. (Dorval, Que.) and potassium fluoride was from BDH Chemicals (Toronto, Ont., Canada). PMA, aluminum chloride ($AlCl_3.6H_2O$), sodium orthovanadate, ATP (disodium salt, prepared by phosphorylation of adenosine), GTP, EDTA, EGTA, PIPES, HEPES, heparin, glutamic acid, BSA (Fraction V), sodium formate and ammonium formate were purchased from Sigma (St. Louis, MO, USA). Hydrogen peroxide (H_2O_2) and highly purified $CaCl_2.4H_2O$ (Suprapur) were obtained from E. Merck (Darmstadt, Germany). ST271 was a gift from the Wellcome Foundation Ltd. (Beckenham, Kent). Sepharose CL-4B was from Pharmacia (Canada)(Dorval, Que., Canada). Silica gel t.l.c. plates (SIL G-25) were from the J.T. Baker Chemical Co. Dowex-1 anion-exchange resin (AG 1-X8, 100-200 mesh, formate form) was obtained from

Bio-Rad Laboratories (Canada) Ltd. (Mississauga, Ont., Canada). Activated silicic acid (Unisil, 100-200 mesh) was from Clarkson Chemical Company Inc. (Williamsport, Pennsylvania). Potato apyrase was prepared as previously described (Molnar and Lorand, 1961).

PMA and ST271 were dissolved in dimethylsulphoxide [final concentration of 0.2% (v/v)].

2.2. *Isolation, labelling and electropermeabilization of platelets*

Human blood from healthy donors were collected into ACD anticoagulant (Aster and Jandl, 1964). Platelet-rich plasma was obtained by centrifugation at 200 *g* for 15 min (37° C) and the platelets were isolated by further centrifugation at 2400 *g* for 15 min (37° C) as described in Haslam and Davidson (1984a). Plastic tubes or siliconized glass tubes and Pasteur pipettes were used for collection, centrifugation, resuspension and incubation of platelets. Platelets were resuspended at 1 - 5 x 10^9 platelets/ml in 5-10 ml of the supernatant plasma (platelet-enriched plasma) and were labelled by incubation with ^{14}C- or ^{3}H-labelled compounds at 37° C, as described below. Labelled platelets were isolated by centrifugation (2400 *g* for 15 min at 37° C) and suspended in Ca^{2+}-free Tyrode's solution (pH 6.5) containing 5 mM PIPES, 0.35% bovine serum albumin, 50 units of heparin/ml and apyrase (30 μg/ml) and incubated for 15 min at 37° C. Platelets were washed by re-centrifugation (1400 *g* for 10 min at 37° C) and resuspended at room temperature at 2 x 10^9 platelets/ml in the same Ca^{2+}-free Tyrode's solution (pH 6.5) without the addition of heparin or

apyrase.

These platelets were permeabilized as described by Haslam and Davidson (1984a). After addition of 5 mM EGTA (K^+ salt, pH 6.5), platelet suspension (2 ml portions) were placed in a 0.2-cm-wide chamber with 10 cm^2 stainless steel electrodes. After using a fine plastic needle to remove the bubbles gently from the chamber, platelet suspension was then permeabilized by 10 electric discharges (1 discharge/s) from capacitors (4.5 µF) charged at 3.0 kV. The permeabilized platelets were immediately cooled to 4°C and the chamber was cooled after each 10 discharges to prevent the temperature of the platelet suspension rising above 24°C during each permeabilization. The permeabilized platelets were isolated on a 20 x 1.5 cm column of Sepharose CL-4B at 4°C to remove released low-M_r compounds. The columns had previously been washed first with 5 ml of 10% BSA (w/v) in a medium (pH 7.4) containing 3.9 mM $MgCl_2$ and the K^+ salts of glutamic acid (160 mM), HEPES (20 mM), EGTA (2.5 mM) and EDTA (2.5 mM), and then with about 150 ml of this medium alone. As soon as the platelets were eluted from the columns in this glutamate-based medium, they were diluted (to 5 x 10^8 platelets/ml or the required platelet count) with the same medium. ATP (Na^+ salt, pH 7.4) was added to a final concentration of 5 mM and this suspension was stored at 0°C until used (within 2 h).

2.3. *Measurement of [^{14}C]5-HT secretion*

When secretion alone was measured (Haslam and Davidson, 1984a), [^{14}C]5-

HT was added to the platelet-enriched plasma (5 x 10^9 platelets/ml) to give a final concentration of 0.25 μCi/ml. After incubation for 20 min at 37° C, labelled platelets were washed and permeabilized and isolated by gel filtration, as above. After addition of ATP, samples (80 μl) of permeabilized platelets (usually 5 x 10^8 platelets/ml) were equilibrated for 15 min at 0° C with 20 μl of any other additions and the $CaCl_2$ required to give a particular pCa value (see below). Solutions of KF and $AlCl_3$ were carefully prepared using plastic tubes and were premixed to allow the formation of Al-F complexes (Goldstein, 1964) before added to the platelet suspension. In experiments using vanadate and H_2O_2, these two compounds were added at the same time when the equilibration started. These mixtures were then transferred to 25° C and further incubated for 10 min. Incubations were terminated by addition of 0.5 ml of 0.15 M KCl containing 1.8% (w/v) paraformaldehyde and 6 mM EDTA at 0° C. After centrifugation at 12 000 x *g* for 1.5 min, the ^{14}C in the supernatant (500 μl) was counted in 8 ml ACS. Secretion of [^{14}C]5-HT was calculated from the ^{14}C found in the supernatants of incubation mixtures and expressed as percentages of the total platelet-bound ^{14}C found in permeabilized platelet suspension lacking $CaCl_2$ and incubated at 0° C. The ^{14}C found in the supernatant from the latter platelets (< 5 ± 0.2%; mean ± S.E. from 20 determinations) was subtracted in all calculations.

In these experiments, the concentrations of $CaCl_2$ required to give appropriate pCa values in the EGTA/EDTA/Mg^{2+} buffer system were calculated by using a computer program based on that of Fabiato and Fabiato (1979). The concentration

of free Mg^{2+} for all samples was adjusted to 5 mM. The total concentrations of $CaCl_2$ and $MgCl_2$ required to give the desired free concentrations of Ca^{2+} and Mg^{2+} in the glutamate-based medium are shown in Table 1. A pCa > 9 was obtained by increasing the concentration of EGTA from 2.5 mM to 12.5 mM in the absence of added $CaCl_2$ (Coorssen *et al.*, 1990). In order to obtain a final pH of 7.4 in the system after mixing with other additions, the pH values of the $CaCl_2$ solutions were adjusted with KOH.

2.4. Measurement of protein phosphorylation

As described previously (Haslam and Davidson, 1984a), [γ-^{32}P]ATP (100 μCi/ml) was added to the suspension of permeabilized platelets (containing 5 mM unlabelled ATP) about 20 min before incubations started. After equilibration and incubation as described above, incubations (final volume 100 μl) were terminated by addition of 0.5 ml of 10% (w/v) trichloroacetic acid. Each protein pellet was separated by centrifugation (12,000 *g* for 4 min) and was dissolved in 75 μl of electrophoresis sample buffer (Laemmli, 1970) containing 30 μl of 1 M NaOH/1 ml, by either standing overnight at 0°C or warming at 37°C for 30-60 min. Platelet protein was analyzed by SDS-polyacrylamide gel electrophoresis using 13% acrylamide (Imaoka *et al.*, 1983). Labelled polypeptides were located by overnight autoradiography on Du Pont Cronex film with fluorescent screens at -60°C and the regions containing pleckstrin (P47) were cut out and counted for ^{32}P in 0.01% (w/v) 4-methylumbelliferone (Cerenkov radiation). Incorporation of ^{32}P into pleckstrin was

Table 1. Total concentrations of $CaCl_2$ and $MgCl_2$ required to give the indicated free concentrations of Ca^{2+} and Mg^{2+} in glutamate-based buffer containing EGTA, EDTA and ATP

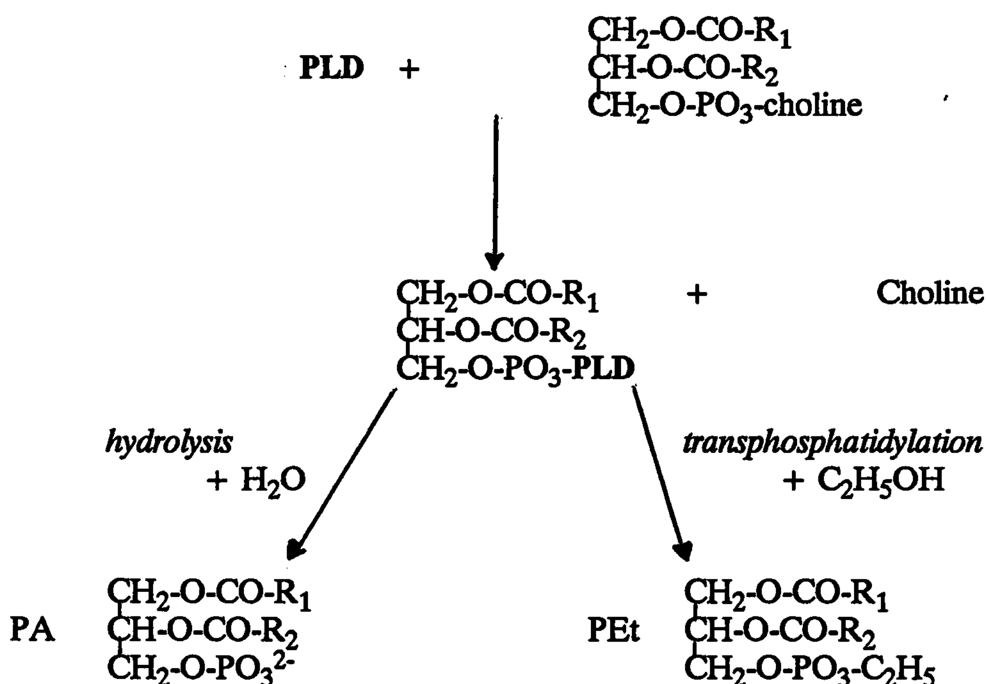
pCa	Total Ca^{2+} (mM)	Free Ca^{2+} (M)	Total Mg^{2+} (mM)	Free Mg^{2+} (mM)
> 9	< 0.1	$< 1.0 \times 10^{-9}$	16.7	5.0
7	1.2	1.0×10^{-7}	12.4	5.0
6	2.3	1.0×10^{-6}	11.9	5.0
5.5	2.6	3.2×10^{-6}	11.7	5.0
5	2.9	1.0×10^{-5}	11.4	5.0
4.5	3.5	3.2×10^{-5}	10.8	5.0

The data in this table were calculated using the binding constants given by Fabiato and Fabiato (1979). The maximum value for the total [Ca^{2+}] is calculated to be 0.1 mM in the absence of added $CaCl_2$, on the assumption that the total platelet Ca^{2+} (80 nmol/mg protein) is released into the medium (Coorssen *et al.*, 1990). In medium containing 2.5 mM EGTA, this would give a pCa > 8. By increasing the EGTA concentration from 2.5 mM to 12.5 mM, a pCa value > 9 was obtained.

expressed as pmol/10^9 platelets.

2.5. *Measurement of PLD activity*

PLD normally hydrolyses phospholipids, such as PC, to generate free choline and PA. However, in the presence of a primary alcohol, PLD catalyses a unique transphosphatidylation reaction in which phosphatidyl moiety is transfered to the alcohol to produce a phosphatidylalcohol that is readily separated from other phospholipids. PLD activity was therefore measured by exploitation of its ability to catalyse the formation of PEt through transphosphatidylation in the presence of ethanol. The decrease in PA formation in the presence of ethanol also indicates the presence of PLD activity. The pathway by which PA and PEt are produced by PLD is thought to be as follows:



2.5.1. Measurement of [^{3}H]PEt and [^{3}H]PA formation. In these experiments, platelets were labelled with both [^{3}H]arachidonic acid and [^{14}C]5-HT. Platelet-enriched plasma (5 x 10^9 platelets/ml) was incubated with 5 µCi of [^{3}H]arachidonic acid/ml for 1 h at 37°C. [^{14}C]5-HT (0.25 µCi/ml) was added during the last 20 min. Labelled platelets were washed and permeabilized and isolated by gel filtration as above. After addition of ATP, samples of permeabilized platelets were equilibrated for 15 min at 0°C in mixtures (final volume of 0.4 ml) containing 0.32 ml of platelet suspension (4 x 10^8 platelets) and 80 µl of any other additions, including an appropriate concentration of $CaCl_2$, with or with out 200 mM ethanol. These mixtures were then transferred to 25°C and further incubated for 10 min. At the end of incubation, 50 µl of suspension was used for measurement of [^{14}C]5-HT secretion as above, and the lipids were extracted from the remainder, as described by Bligh and Dyer (1959). Solvent was removed by centrifugation under vacuum (Savant), and the lipid was redissolved in 50 µl of chloroform containing unlabelled PEt and PA standards. [^{3}H]PEt and [^{3}H]PA were then isolated by t.l.c. (Bocckino et al., 1987). The t.l.c. solvent for the separation of [^{3}H]PEt and [^{3}H]PA, contained ethyl acetate/2,2,4-trimethylpentane/acetic acid (9:5:2 by vol.). Samples and standards (prepared as below) were applied to Silica gel plates (SIL G-25) under nitrogen and, after development for 2 h, plates were exposed to iodine vapour. The areas containing PEt (R_F = 0.44) and PA (R_F = 0.35) were scraped into vials containing 0.5 ml of methanol and 50 µl of acetic acid. ^{3}H was counted in 8 ml ACS. Results were expressed as dpm/10^9 platelets after values for [^{3}H]PEt or [^{3}H]PA found in

samples from platelet suspension stored at 0°C were subtracted.

2.5.2. Preparation of PEt/PA standard. PEt and PA standard was prepared as described by Kobayashi and Kanfer (1987). Approximately 25 mg of egg phosphatidylcholine (PC) was dissolved in 17.5 ml of reaction medium containing sodium acetate (0.1 M), $CaCl_2$ (37.5 mM), SDS (0.5 mM) and ethanol (1.5 M), and was thoroughly mixed in a water bath-sonicator. After addition of 5 mg of cabbage phospholipase D dissolved in 2.5 ml of reaction medium, the mixtures were incubated for 2 h at 30°C. The reaction was terminated by addition of 20 ml of $CHCl_3$/methanol (2:1) and lipids were extracted by centrifugation at 60 *g* for 10 min. The extracts were pooled and dried by removing the solvent in a Rotovap, and then redissolved in 4 ml $CHCl_3$. Each 1 ml of lipid solution was applied to the column containing 1 g of silicic acid (Unisil, 100-200 mesh). The column was eluted first with 10 ml of $CHCl_3$:methanol (97:3 by vol.), and then with 10 ml of $CHCl_3$:methanol (47:3 by vol.). The latter eluate containing PEt and PA was collected, dried in a Rotovap and redissolved in 10 ml of $CHCl_3$. After t.l.c. of 10 µl, 25 µl and 50 µl in ethyl acetate/2,2,4-trimethylpentane/acetic acid (9:5:2 by vol.), this solution was diluted with $CHCl_3$ so that 50 µl gave readily visible spots on t.l.c. Aliquots of this PEt/PA standard were stored at -20°C in scintillation vials with foil-lined caps.

2.6. *Measurement of PLC activity*

In permeabilized platelets, it has been found that PLC mainly hydrolyses

phosphatidylinositol 4-phosphate (PIP) to generate DAG and IP_2 (Culty *et al.*, 1988). There was much less hydrolysis of phosphatidylinositol 4,5-bisphosphate (PIP_2) to IP_3 and of phosphatidylinositol to IP (Culty *et al.*, 1988). PLC activity was measured by the formation of DAG or of inositol phosphates.

2.6.1. Measurement of [^{3}H]DAG formation. [^{3}H]DAG was also measured after isolation by t.l.c. (Rittenhouse-Simmons, 1979). Before the extraction of lipid, [^{14}C]DAG (about 600 dpm) was added in each sample to permit calculation of the recovery of the compound. After Bligh and Dyer extraction, solvent was removed as before and the lipid was dissolved in 50 µl of chloroform containing D-1,2-diolein (50 µg) and triolein (50 µg) as standards. The chromatographic solvent contained benzene/diethyl ether/ethanol/concentrated NH_3 (100:80:4:0.4, by vol.). Samples and standards were applied to plates (SIL G-25) as above. The areas containing DAG were detected with iodine and were scraped into vials containing 0.5 ml of methanol and 50 µl of acetic acid. ^{3}H and ^{14}C were counted in 8 ml ACS. Results were corrected for the recovery of [^{14}C]DAG and were expressed as dpm/10^9 platelets after values for [^{3}H]DAG found in samples from platelet suspension stored at 0°C were subtracted.

2.6.2. Measurement of [^{3}H]inositol phosphate formation. After isolation from platelet-rich plasma, platelets were resuspended and washed in a modified Tyrode's solution (Culty *et al.*, 1988). Then, platelets were labelled with [^{3}H]inositol (20

μCi/ml) in a Tyrode's solution in which 2 mM $MgCl_2$ and 5.6 mM glucose were relpaced by 2 mM $MnCl_2$ and 0.56 mM glucose (Culty *et al.*, 1988). After 2 h incubation at 37° C, platelets were washed and permeabilized as above. Samples (400 μl) of permeabilized platelet supernatant (0.6 - 1 x 10^9 platelets/ml) were equilibrated for 15 min at 0° C with 100 μl of other additions (including the $CaCl_2$ required to give a particular pCa value), and then incubated for 10 min at 25° C before addition of 250 μl of 30% (w/v) trichloroacetic acid. Samples were centrifuged and 650 μl of supernatant was removed and neutralized with NaOH, using bromthymol blue as an indicator. Samples were stored at -20° C until applied to Dowex-1 anion-exchange resin (AG 1-X8, 100-200 mesh). Columns containing 2.5 ml of Dowex-1 resin (1:1 in H_2O) were prepared. Samples were diluted to 5 ml with H_2O before they were loaded on the columns. First, [^{3}H]inositol was eluted with 3 x 5 ml of H_20, and [^{3}H]glycerophosphoinositol with 2 x 5 ml of 60 mM sodium formate/5 mM disodium tetraborate. [^{3}H]IP was then eluted with 3 x 5 ml of 0.15 M ammonium formate in 0.1 M formic acid, followed by [^{3}H]IP_2 with 3 x 5 ml of 0.4 M ammonium formate in 0.1 M formic acid, and [^{3}H]IP_3 and [^{3}H]IP_4 with 2 x 4.5 ml of 1.2 M ammonium formate in 0.1 M formic acid. Each fraction was collected and adjusted to contain 1 M ammonium formate before addition of 15 ml of ACS for counting of ^{3}H. Results were expressed as dpm/10^9 platelets after the subtraction of blank values obtained from platelet suspension stored at 0° C.

2.7. *Analysis of the aluminum content of solutions*

In order to determine the aluminum content in the glutamate-based buffer with and without the KF, mock samples containing all required additions at pCa 6 except for platelets were analysed by ICP-MS.

2.8. *Statistics*

Incubations for measurement of [^{14}C]5-HT secretion, [^{3}H]PEt, [^{3}H]PA, [^{3}H]DAG and [^{3}H]inositol phosphate formation were performed in triplicate. Mean values ± standard error (S.E.) are given from triplicate samples within experiments. S.E. represents the standard error of the mean (S.E.M.) in the measurement of [^{14}C]5-HT secretion, or the standard error of the difference (S.E.D.) in measurements of the formation of phospholipids metabolites, from which initial values (mean ± S.E.) were subtracted. Incubations for measurement of protein phosphorylation were performed in duplicate; mean values ± range are given. The significance of differences within an experiment containing replicate (triplicate) samples was determined by two-sided unpaired *t*-tests. Pooled results from different experiments are given as means ± S.E. and the number of experiments is indicated; the significance of differences was then determined by two-sided paired *t*-tests.

Standard Error of the Mean

$$\text{S.E.M.}(x) = [\ \Sigma\ (x-\bar{x})^2/n-1]^{1/2}\ /n^{1/2}$$

Standard Error of the Difference

$$S.E.D.(\bar{x}-\bar{y}) = [(S.E.M._x)^2 + (S.E.M._y)^2]^{1/2}$$

Unpaired *t*-test

$$t = (\bar{x}_T - \bar{x}_c)[n_T n_c/(n_T+n_c)]^{1/2}/s$$

t = t distribution with $n_T + n_c - 2$ degrees of freedom

$x_T - x_c$ = difference in independent sample means for treatment and control groups

$s^2 = [\ \Sigma(x_T - \bar{x}_T)^2 + \Sigma(x_c - \bar{x}_c)^2\ ]/(n_T + n_c - 2)$

T = number of treatment samples

c = number of control samples

$(x_T - \bar{x}_T)^2$ = sum of squares about the mean of the treatment group

$(x_c - \bar{x}_c)^2$ = sum of squares about the mean of the control group

Paired *t*-test

$t = \bar{d}n^{1/2}/s_d$ $\qquad$ $s_d{}^2 = [\Sigma(d-\bar{d})^2]/n-1$

t = t distribution with n-1 degree of freedom

n = number of paired observations

d = difference for each of n paired observations

$\bar{d}$ = mean of sample differences

s_d = sample standard deviation of the differences

3. RESULTS

3.1. *Effects of $KF/AlCl_3$ on secretion*

3.1.1. Effects of different concentrations of $KF/AlCl_3$ on Ca^{2+}-dependent [^{14}C]5-HT secretion. In this study, [^{14}C]5-HT was used as a marker for the secretion of platelet dense granule constituents. Permeabilized human platelets contained about $1.6 \pm 0.1 \times 10^5$ dpm of [^{14}C]5-HT/10^9 platelets (mean ± S.E. from 20 determinations). To determine the optimal concentrations of $KF/AlCl_3$ and Ca^{2+} required for 5-HT secretion, samples of permeabilized platelets were incubated with different concentrations of KF and $AlCl_3$ at various buffered Ca^{2+} concentrations. In Fig. 1A, Ca^{2+}-dependent [^{14}C]5-HT secretion induced by $KF/AlCl_3$ was observed. In the absence of Ca^{2+} (pCa > 9), $KF/AlCl_3$ did not cause [^{14}C]5-HT secretion, and at a buffered pCa of 7, low concentrations of $KF/AlCl_3$ (4 mM and 4 µM, respectively) also did not lead to [^{14}C]5-HT secretion. However, higher concentrations of $KF/AlCl_3$ at pCa 7 caused a 15-20% release of [^{14}C]5-HT from platelet dense granules. On increasing the Ca^{2+} concentration to pCa 6, which causes some (about 20%) secretion, $KF/AlCl_3$ induced a more marked concentration-dependent effect on [^{14}C]5-HT secretion. A maximum secretion of 40-66% of [^{14}C]5-HT (mean ± S.E., 53 ± 3% from 12 expts.) was observed in the presence of

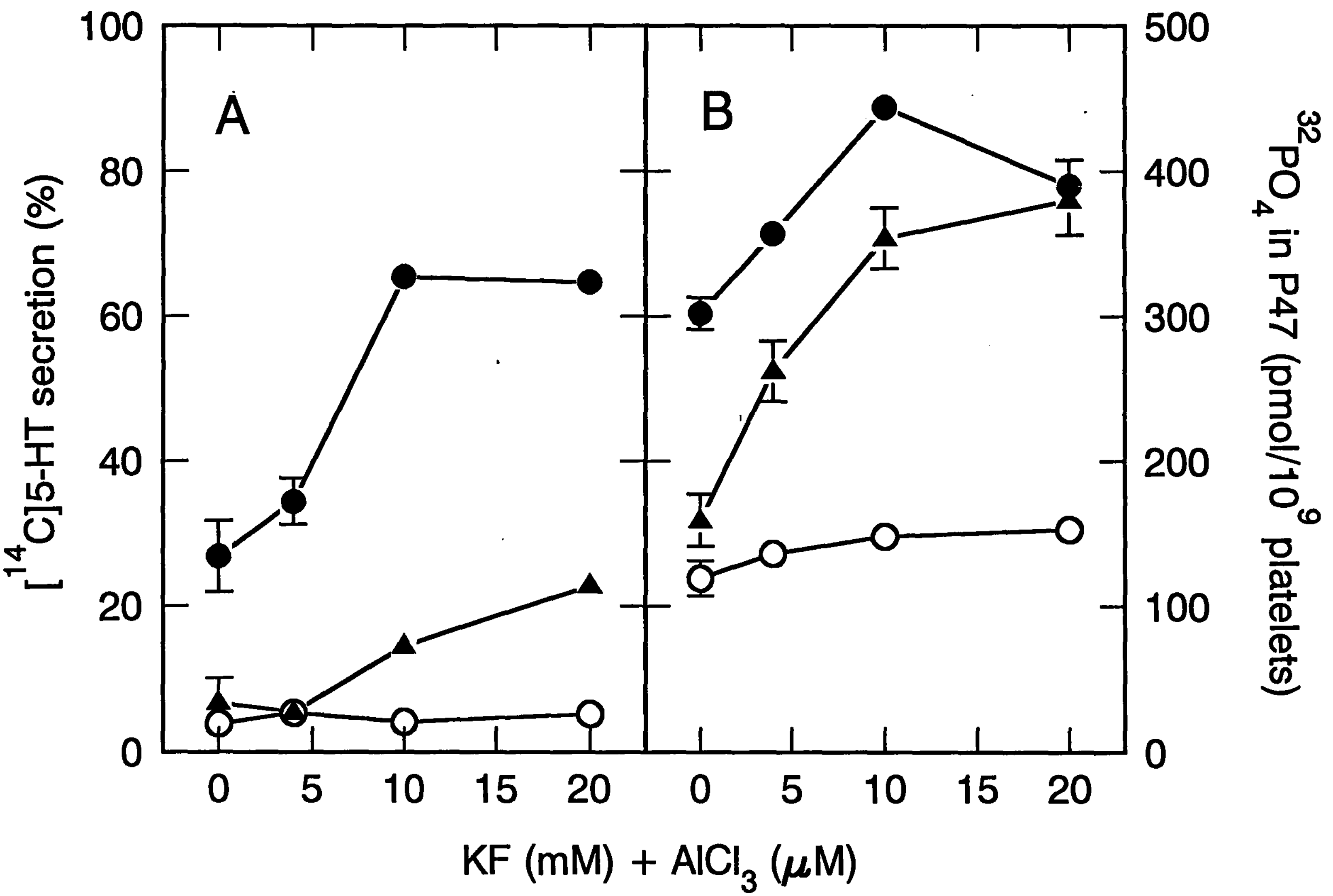
10 mM KF and 10 μM $AlCl_3$ (Fig. 1A).

It was found in previous studies that the addition of $AlCl_3$ potentiated the effects of NaF by formation of the AlF_4^- ion in intact human platelets (Rendu *et al.*, 1990). Experiments were carried out to study the effects of aluminum ions on fluoride-induced [^{14}C]5-HT secretion at pCa 6. The effects of varying concentrations of KF on [^{14}C]5-HT secretion were not potentiated by the addition of 10 μM $AlCl_3$ (Table 2). The addition of 1-10 μM $AlCl_3$ alone had no effects on secretion, and did not show significant effects on the secretion caused by 10 mM KF (Table 2). These results suggested that either KF alone could cause [^{14}C]5-HT secretion without the addition of $AlCl_3$, or that Al^{3+} contamination in the glutamate-based buffer may account for these results. Results from ICP-MS demonstrated that glutamate-based buffer at pCa 6 contained 250 ppb aluminum, which is equivalent to 9.4 μM. After addition of 10 mM KF in this buffer system, the aluminum content was 350 ppb, equivalent to 13.2 μM, indicating that 10 mM KF itself contains approximately 4 μM aluminum. In the succeeding experiments, a combination of KF in the mM concentration range with $AlCl_3$ at the same μM concentration was used based on the knowledge that formation of AlF_4^- should occur under these conditions (Goldstein, 1964), whether Al^{3+} contamination is present or not.

3.1.2. Comparison of the effects of KF/$AlCl_3$ and of GTPγS and PMA on [^{14}C]5-HT secretion. To understand the mechanism of KF/$AlCl_3$ action, experiments were carried out to compare the effects of KF/$AlCl_3$ with those of GTPγS, which is a

Figure 1. Effects of different concentrations of $KF/AlCl_3$ on $[^{14}C]$5-HT secretion and P47 phosphorylation in permeabilized platelets at various buffered Ca^{2+} concentrations

Samples of permeabilized platelets containing $[^{14}C]$5-HT and when required, $[\gamma\text{-}^{32}P]$ATP, were equilibrated (15 min at 0°C) and then incubated (10 min at 25°C) with the indicated concentrations of KF and $AlCl_3$ in the absence of Ca^{2+} (pCa > 9, ○) or in the presence of Ca^{2+} (pCa 7, ▼; pCa 6, ●). Secretion of $[^{14}C]$5-HT (triplicate samples) (A) and the phosphorylation of P47 (duplicate samples) (B) were determined; values are means ± S.E. or means ± range, respectively. (C) Proteins were resolved by SDS/polyacrylamide-gel electrophoresis; an autoradiograph of the dried gel is shown. Other details are given in the Experimental section.



A
B
100
80
60
40
20
0
$[^{14}C]$5-HT secretion (%)
500
400
300
200
100
0
$^{32}PO_4$ in P47 (pmol/10^9 platelets)
0
5
10
15
20
KF (mM) + $AlCl_3$ (μM)

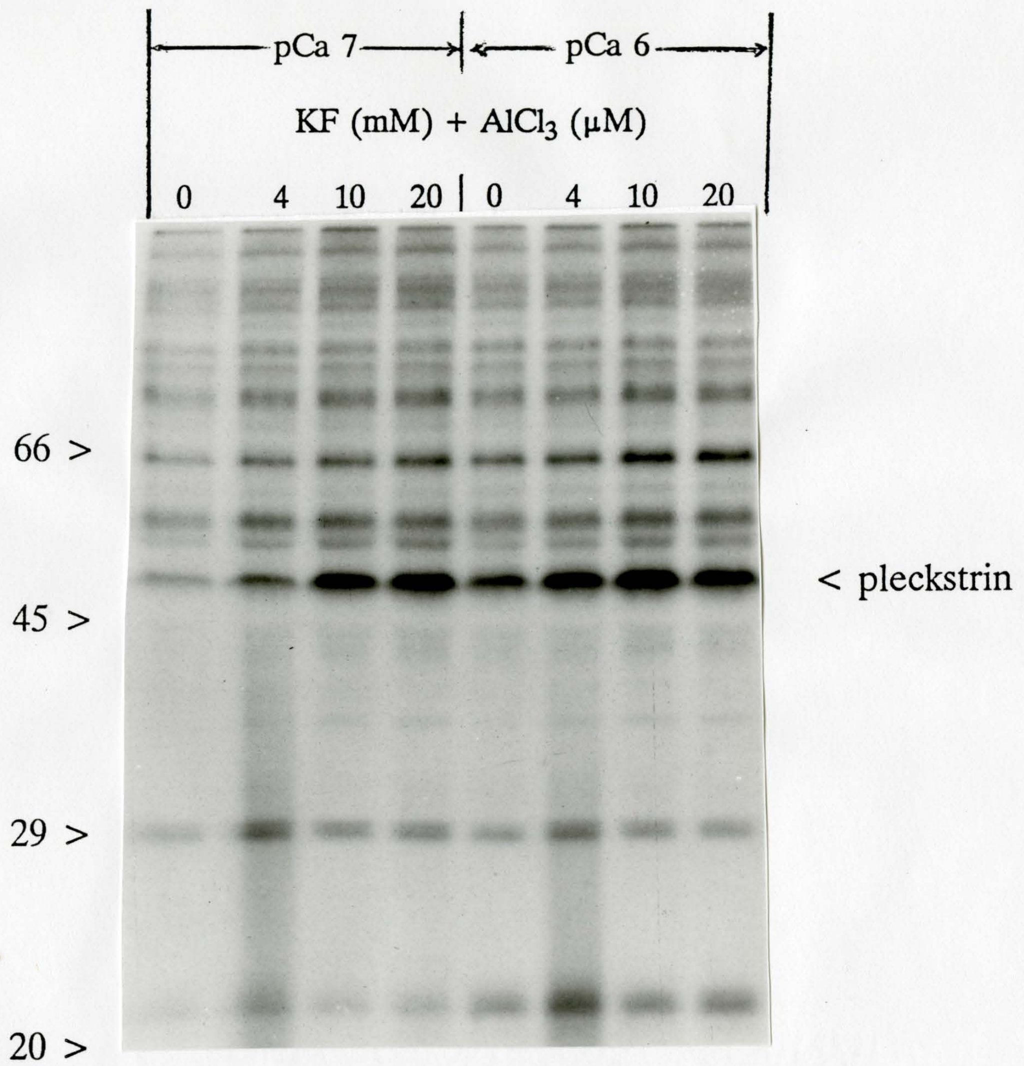

C
pCa 7
pCa 6
KF (mM) + AlCl3 (μM)
0
4
10
20
0
4
10
20
66 >
45 >
29 >
20 >
< pleckstrin

Table 2. Effects of KF or $AlCl_3$ on secretion from permeabilized platelets at pCa 6

Samples of permeabilized platelets containing [^{14}C]5-HT were equilibrated (15 min at 0° C) and incubated (10 min at 25° C) with the indicated concentrations of of KF, in the absence or presence of the indicated concentrations of $AlCl_3$, all at pCa 6. Secretion of [^{14}C]5-HT was determined; values are means ± S.E. from triplicate samples.

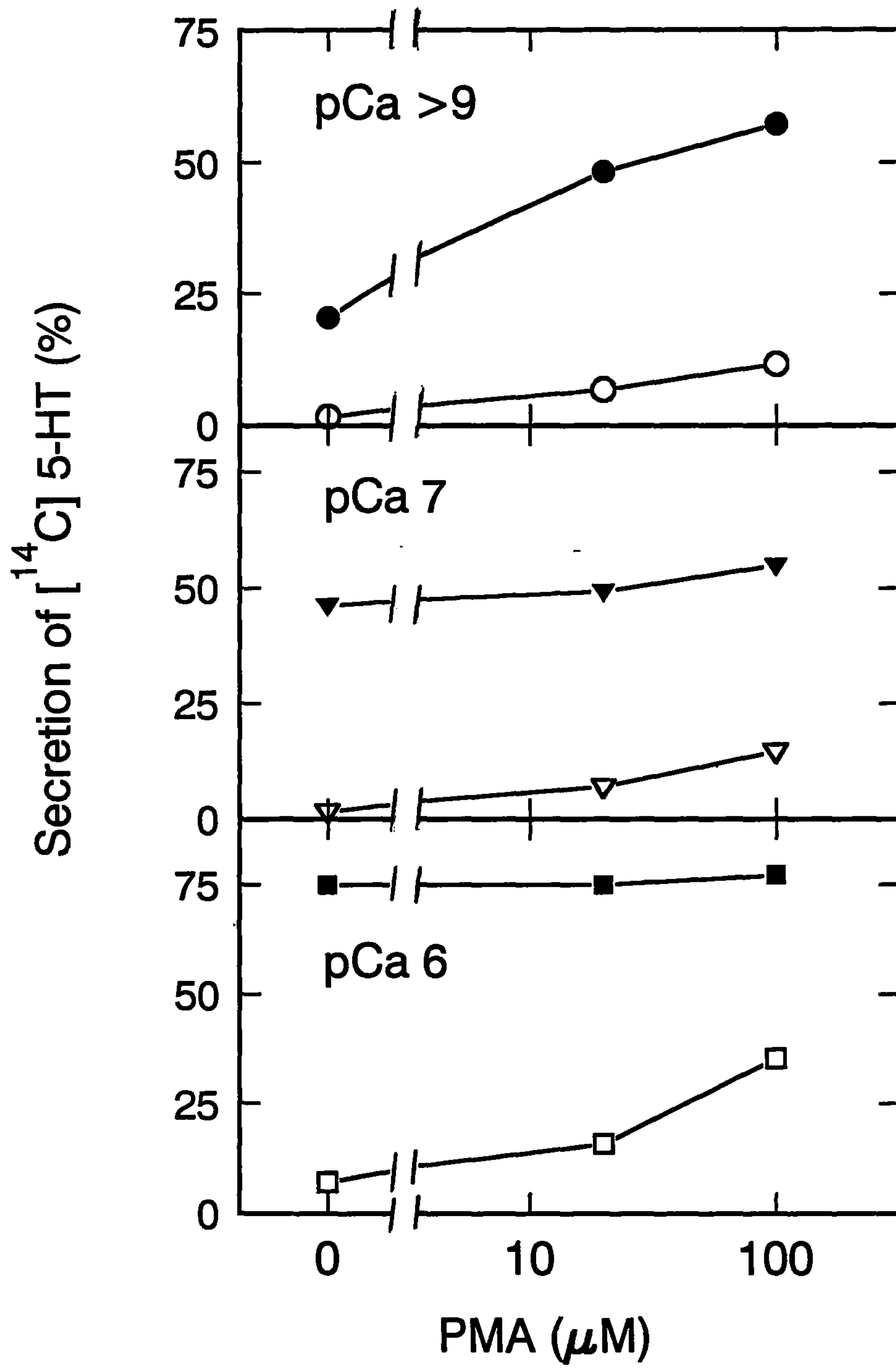
	$[^{14}C]$5-HT secretion (%)	
Additions	- 10 μM $AlCl_3$	+ 10 μM $AlCl_3$
None	23 ± 0	27 ± 3
1 mM KF	35 ± 4	30 ± 5
4 mM KF	49 ± 2	47 ± 1
10 mM KF	66 ± 1	66 ± 4
20 mM KF	59 ± 1	60 ± 1
	- 10 mM KF	+ 10 mM KF
None	23 ± 0	66 ± 1
1 μM $AlCl_3$	24 ± 0	68 ± 2
10 μM $AlCl_3$	27 ± 3	66 ± 4

useful tool to demonstrate G protein-dependent pathways, and with those of PMA, which is a PKC activator. Previous studies in this laboratory showed that GTPγS not only greatly enhanced the Ca^{2+} sensitivity of the $[^{14}C]$5-HT secretion, but also could induce a Ca^{2+}-independent secretion which was potentiated by PMA (Coorssen *et al.*, 1990; Coorssen and Haslam, 1993). To confirm these results, permeabilized platelets were incubated with GTPγS or PMA at various buffered Ca^{2+} concentrations as shown in Fig. 2. At pCa >9, GTPγS (100 μM) caused a Ca^{2+}-independent secretion of 21% of $[^{14}C]$5-HT by itself, whereas PMA (100 nM) alone also induced 12% $[^{14}C]$5-HT secretion. When added together, these stimuli caused a 57% secretion that was 1.8-fold greater than the sum of their individual effects. At pCa 7, GTPγS (100 μM) alone caused the secretion of 46% of $[^{14}C]$5-HT and at pCa 6, GTPγS (100 μM) caused a maximal secretion of 75% of $[^{14}C]$5-HT. PMA was less effective than GTPγS; PMA (100 nM) alone only induced a 15% $[^{14}C]$5-HT secretion at pCa 7 and a 35% secretion at pCa 6. GTPγS and PMA did not show synergistic effects on secretion at pCa 7 or 6. These results are fully consistent with the earlier studies in this laboratory (Coorssen *et al.*, 1990).

To determine whether KF/$AlCl_3$ interacts with PMA similarly to GTPγS, permeabilized platelets were incubated with both PMA and KF/$AlCl_3$. As shown in Fig. 3A, KF/$AlCl_3$ increased PMA-induced $[^{14}C]$5-HT secretion at pCa >9 by about 52 ± 7% (mean ± S.E., 5 expts.; $P < 0.01$). The effect of KF/$AlCl_3$ with PMA was much weaker than the synergistic effect of GTPγS with PMA (Fig. 2). At pCa 7 and 6, KF/$AlCl_3$ also increased secretion in the presence of PMA but these effects were

Figure 2. Effects of GTPγS and PMA on $[^{14}C]$5-HT secretion from permeabilized platelets at various buffered Ca^{2+} concentrations

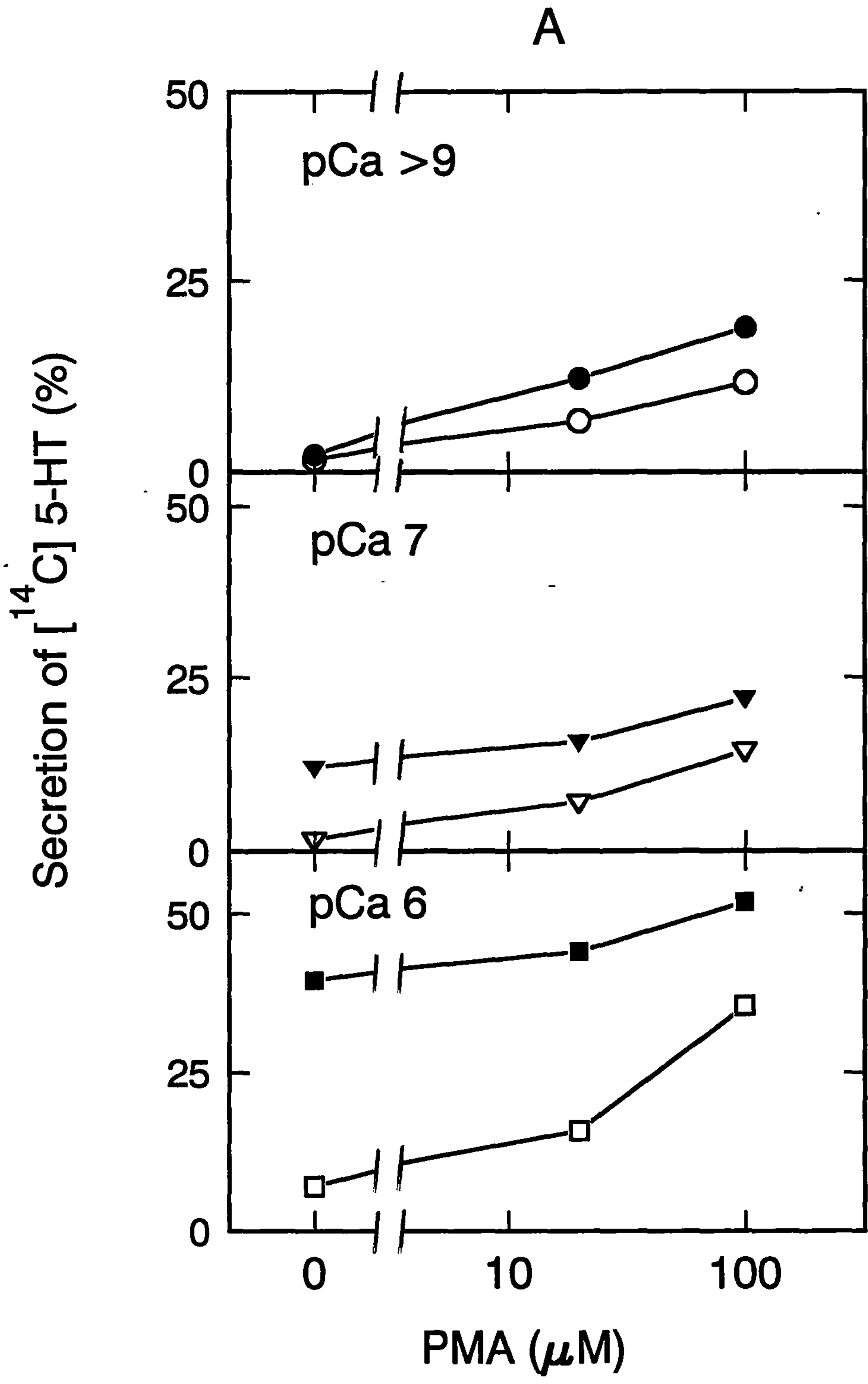
Samples of permeabilized platelets containing $[^{14}C]$5-HT were equilibrated (15 min at 0°C) and then incubated (10 min at 25°C) with the indicated concentrations of PMA, and either no other addition (open symbols), or 100 μM GTPγS (solid symbols) at pCa >9, pCa 7 and pCa 6. Secretion of $[^{14}C]$5- HT was determined from triplicate samples; values are means ± S.E. (The error bars in this and some other figures are not shown because they would be superimposed on the symbols.)

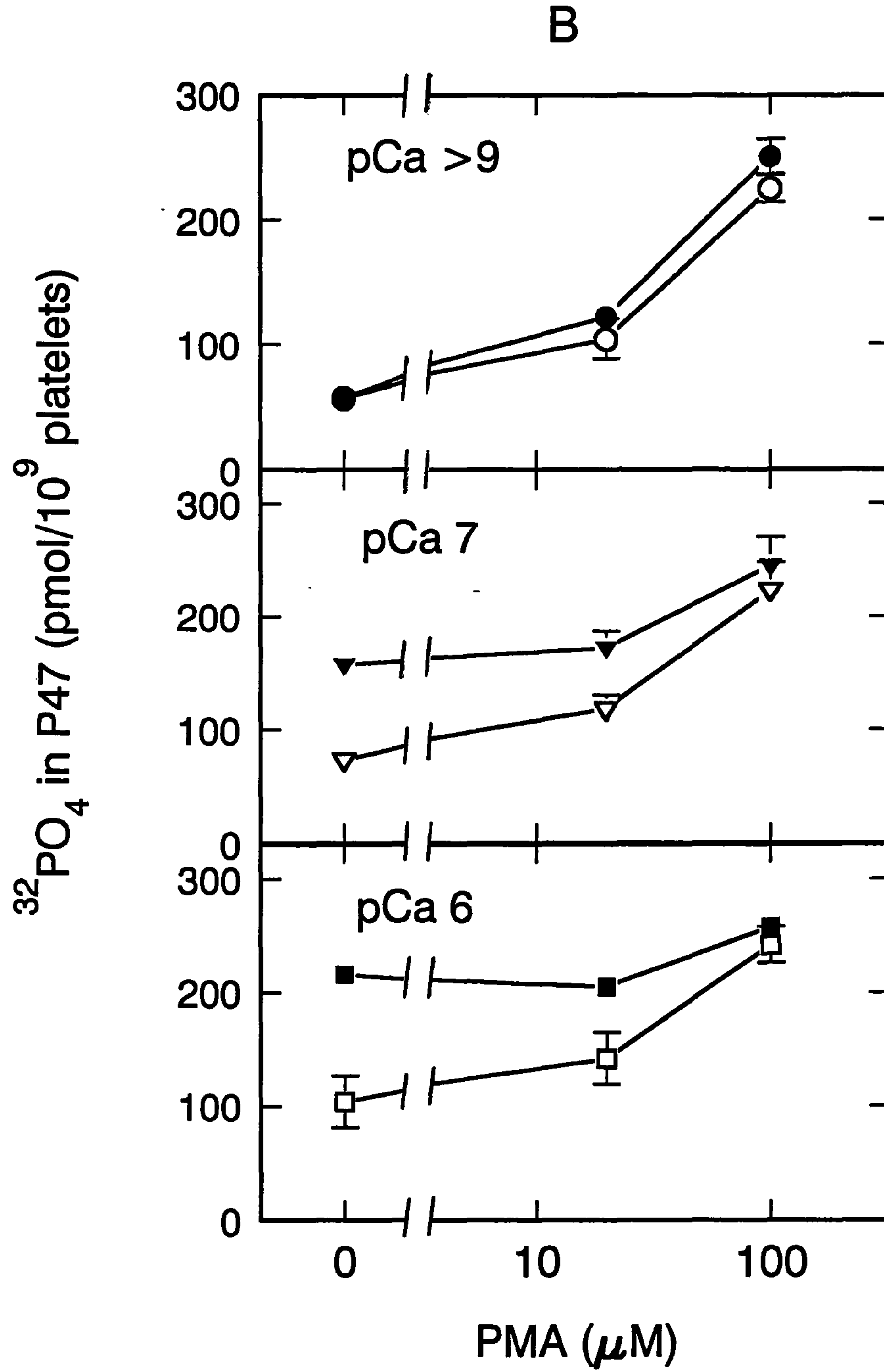


75
50
25
0
pCa >9
75
50
25
0
pCa 7
75
50
25
0
pCa 6
Secretion of [14C] 5-HT (%)
0
10
100
PMA (μM)

Figure 3. Effects of $KF/AlCl_3$ on PMA-induced [^{14}C]5-HT secretion and P47 phosphorylation in permeabilized platelets at various buffered Ca^{2+} concentrations

Samples of permeabilized platelets containing [^{14}C]5-HT were equilibrated (15 min at 0°C) and then incubated (10 min at 25°C) with the indicated concentrations of PMA, and either no other addition (open symbols), or 10 mM KF + 10 μM $AlCl_3$ (solid symbols) at pCa>9, pCa 7 and pCa 6. Secretion of [^{14}C]5-HT (triplicate samples) (A) and the phosphorylation of P47 (duplicate samples) (B) were determined; values are means ± S.E. or means ± range, respectively. These results are from the same experiment as Fig. 2.


A
pCa >9
pCa 7
pCa 6
50
25
0
50
25
0
50
25
0
0
10
100
PMA (μM)
Secretion of [14C] 5-HT (%)


B
pCa >9
pCa 7
pCa 6
300
200
100
0
$^{32}PO_4$ in P47 (pmol/10^9 platelets)
0
10
100
PMA (μM)

less than additive (Fig. 3A).

3.1.3. Effects of $KF/AlCl_3$ on GTPγS-induced [^{14}C]5-HT secretion. Although $KF/AlCl_3$ alone did not cause [^{14}C]5-HT secretion at pCa > 9, this addition had inhibitory effects on GTPγS-stimulated [^{14}C]5-HT secretion (Table 3). With 10 mM KF plus 10 μM $AlCl_3$ and 100 μM GTPγS, the inhibition of secretion amounted to 31 ± 4% (mean ± S.E., 6 expts.), which was significant in a two-sided paired *t*-test ($P < 0.01$). At pCa 7, $KF/AlCl_3$ inhibited GTPγS-induced secretion by 24 ± 7% (mean ± S.E., 3 expts.; $P < 0.02$), whereas $KF/AlCl_3$ only inhibited GTPγS-induced secretion at pCa 6 by 6 ± 1% (mean ± S.E., 4 expts.; $P < 0.02$). As shown in Fig.4, at pCa > 9, $KF/AlCl_3$ not only inhibited secretion induced by GTPγS alone in a concentration-dependent manner, but also inhibited the secretion induced by GTPγS plus PMA. Maximum inhibitions of 42% and 27%, respectively, were obtained with 20 mM KF plus 20 μM $AlCl_3$. Fig. 5 illustrates the concentration-dependent inhibitory effect of $KF/AlCl_3$ on GTPγS-induced [^{14}C]5-HT secretion at pCa 6. The maximum inhibition caused by 20 mM KF with 20 μM $AlCl_3$ amounted to 16%.

3.2. Effects of $KF/AlCl_3$ on pleckstrin (P47) phosphorylation

In addition to Ca^{2+}, PKC is known to play an important role in inducing exocytosis (Nishizuka, 1984). In permeabilized platelets, PKC activation in combination with either Ca^{2+} or GTPγS is essential to cause the near maximal secretion of both dense and α-granule constituents (Haslam and Coorssen, 1993).

Table 3. Effects of $KF/AlCl_3$ on GTPγS-induced secretion and pleckstrin phosphorylation in permeabilized platelets

Samples of permeabilized platelets containing [^{14}C]5-HT and when required, [γ-^{32}P]ATP, were equilibrated (15 min at 0°C) and incubated (10 min at 25°C) with the indicated Ca^{2+} buffers and additions of KF (10 mM) + $AlCl_3$ (10 μM) or GTPγS (100 μM). Secretion of [^{14}C]5-HT (triplicate samples) and the phosphorylation of pleckstrin (duplicate samples) were determined; values are means ± S.E. or means ± range, respectively. The significance of the effects of KF + $AlCl_3$ was evaluated by two-sided unpaired *t*-tests ($^{*}P < 0.005$).

pCa	Additions	Secretion of $[^{14}C]$5-HT (%)	Phosphorylation of pleckstrin (pmol $^{32}PO_4/10^9$ platelets)
> 9	None	2 ± 0	57 ± 4
	KF + $AlCl_3$	2 ± 0	57 ± 3
	GTPγS	21 ± 1	165 ± 9
	GTPγS + KF + $AlCl_3$	17 ± 1	128 ± 4
7.0	None	2 ± 1	74 ± 2
	KF + $AlCl_3$	12 ± 0*	157 ± 4
	GTPγS	46 ± 2	220 ± 11
	GTPγS + KF + $AlCl_3$	35 ± 0*	235 ± 29
6.0	None	7 ± 1	104 ± 23
	KF + $AlCl_3$	40 ± 2*	216 ± 3
	GTPγS	75 ± 0	194 ± 8
	GTPγS + KF + $AlCl_3$	72 ± 1	229 ± 15

Figure 4. Effects of different concentrations of KF/$AlCl_3$ on GTPγS- and GTPγS plus PMA-induced [^{14}C]5-HT secretion from permeabilized platelets at pCa >9

Samples of permeabilized platelets containing [^{14}C]5-HT were equilibrated (15 min at 0°C) and incubated (10 min at 25°C) with the indicated concentrations of KF + $AlCl_3$, and either no other addition (O), 100 μM GTPγS (■) or 100 μM GTPγS + 100 nM PMA (▼), all in the absence of Ca^{2+} (pCa > 9). Secretion of [^{14}C]5-HT was determined; values are means ± S.E. from triplicate samples.

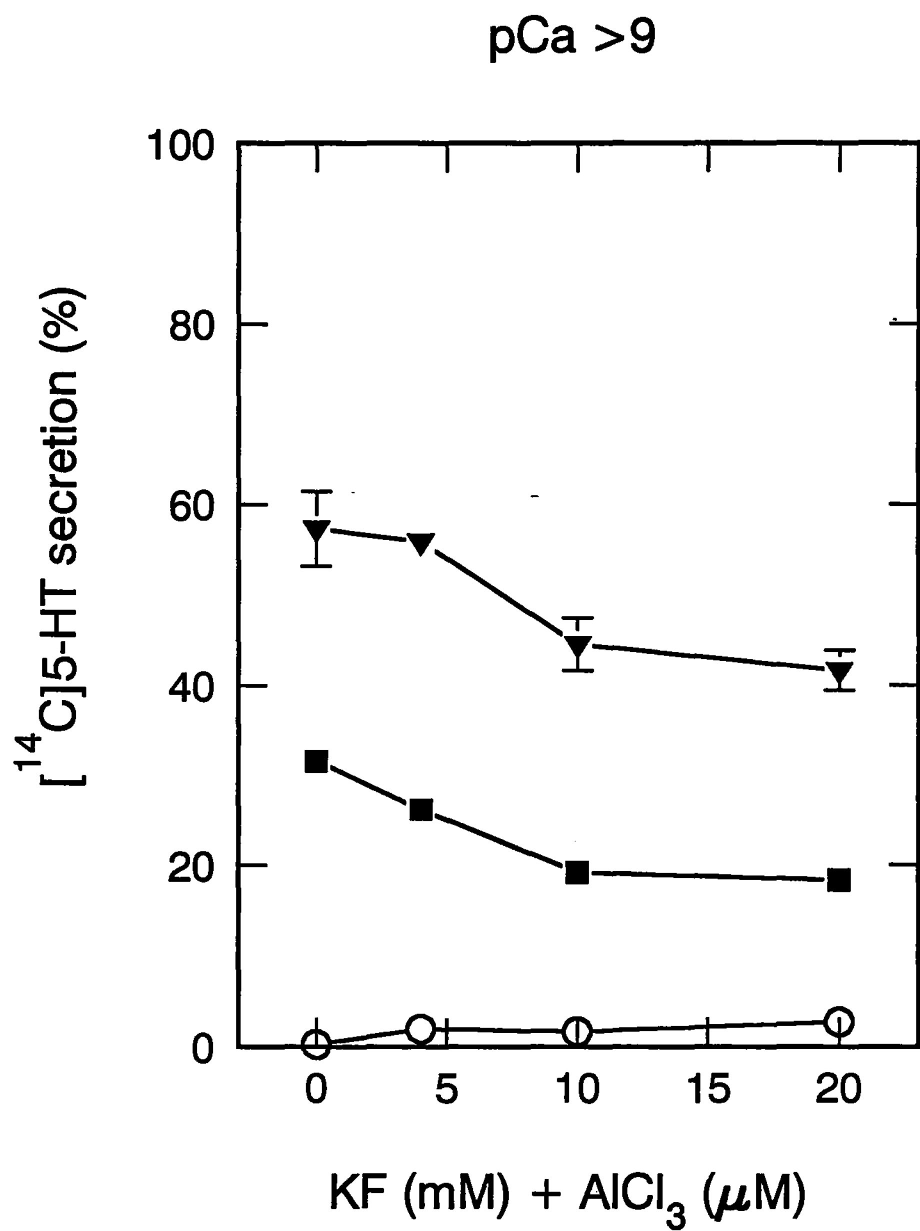
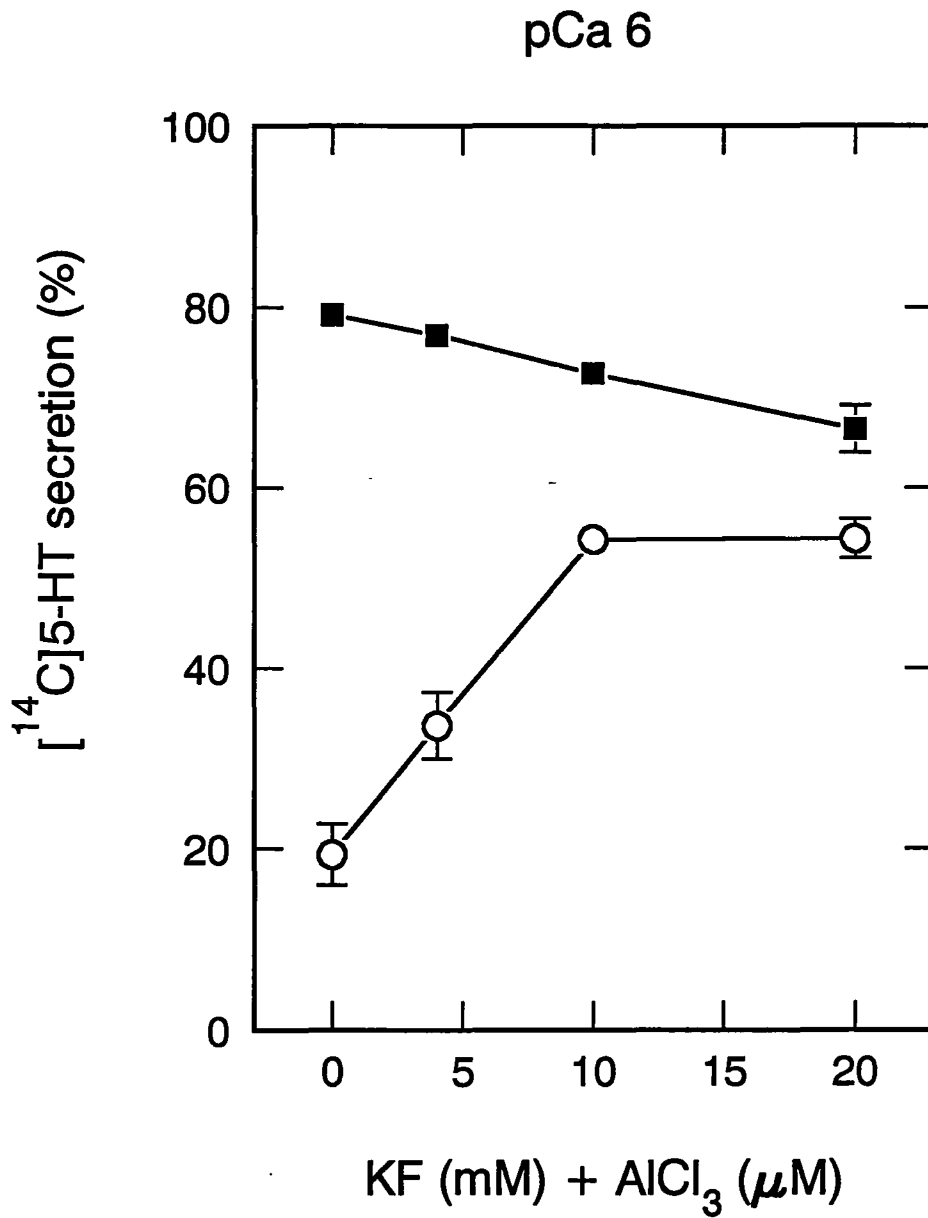

pCa >9
100
80
60
40
20
0
0
5
10
15
20
[14C]5-HT secretion (%)
KF (mM) + AlCl3 (μM)

Figure 5. Effects of different concentrations of $KF/AlCl_3$ on GTPγS-induced $[^{14}C]$5-HT secretion from permeabilized platelets at pCa 6

Samples of permeabilized platelets containing $[^{14}C]$5-HT were equilibrated (15 min at 0°C) and incubated (10 min at 25°C) with the indicated concentrations of KF + $AlCl_3$, and either no other addition (O) or 100 μM GTPγS (■), all in the presence of Ca^{2+} (pCa 6). Secretion of $[^{14}C]$5-HT was determined; values are means ± S.E. from triplicate samples.


pCa 6
100
80
60
40
20
0
0
5
10
15
20
[14C]5-HT secretion (%)
KF (mM) + AlCl3 (μM)

Therefore, I studied PKC activation in KF/$AlCl_3$-stimulated permeabilized platelets.

3.2.1. Effects of different concentrations of KF/$AlCl_3$ on pleckstrin phosphorylation.

It has been shown previously in intact human platelets that NaF stimulates protein phosphorylation (Nakamura *et al.*, 1988). The phosphorylation of pleckstrin (P47), which is the major substrate of PKC, serves as an index of PKC activity during secretion. In the present studies, incubation of permeabilized platelets with KF/$AlCl_3$ in the presence of [γ-^{32}P]ATP caused a marked increase in ^{32}P-labelling of pleckstrin in the presence of Ca^{2+} (pCa 7 and 6), as shown in Fig. 1B and C. At pCa 7, 10 mM KF plus 10 μM $AlCl_3$ caused incorporation of ^{32}P into P47 equivalent to 2.2 $\pm$ 0.1-fold (mean $\pm$ range, 2 expts.) that in the absence of KF/$AlCl_3$; whereas at pCa 6, it caused a 1.6 $\pm$ 0.2-fold (mean $\pm$ S.E., 4 expts.) increase in labelling relative to that in the absence of KF/$AlCl_3$. However, KF/$AlCl_3$ had no significant effect on P47 phosphorylation in the absence of Ca^{2+} (pCa > 9) (Fig. 1B).

3.2.2. Effects of KF/$AlCl_3$ on PMA- or GTPγS-induced pleckstrin phosphorylation.

As shown in Fig. 3B, in the absence of Ca^{2+} (pCa >9), KF/$AlCl_3$ had no significant effects on PMA-induced pleckstrin phosphorylation. A maximum incorporation of ^{32}P into P47 equivalent to 4-fold the control was seen with by 100 nM PMA. In the presence of Ca^{2+} (pCa 7 and 6), the effects of KF/$AlCl_3$ and PMA were not additive. The same maximal effect was observed with KF/$AlCl_3$, PMA and KF/$AlCl_3$ plus PMA at pCa 6 (Fig. 3B). At pCa >9, KF/$AlCl_3$ slightly inhibited the effect of GTPγS on

pleckstrin phosphorylation (Table 3), but at pCa 7 and 6, the effects of GTPγS on pleckstrin phosphorylation were not affected by KF/$AlCl_3$. Addition of Ca^{2+} ions markedly increased the phosphorylation of pleckstrin by KF/$AlCl_3$ and also slightly increased the phosphorylation of pleckstrin by GTPγS, but never to the level seen with 100 nM PMA alone.

3.3. Effects of KF/$AlCl_3$ on phospholipase D (PLD) activity

3.3.1. Effects of KF/$AlCl_3$ on PLD activity at pCa >9. Previous studies in this laboratory have shown that secretion correlated well with the activation of PLD (Coorssen and Haslam, 1993). Here, I sought to determine whether the secretion caused by KF/$AlCl_3$ was also mediated by the activation of PLD. The activity of PLD was measured by the formation of [^{3}H]phosphatidylethanol ([^{3}H]PEt) in the presence of ethanol, as a result of transphosphatidylation. The decrease in formation of [^{3}H]phosphatidic acid ([^{3}H]PA) in the presence of ethanol provided an additional measure of PLD activity.

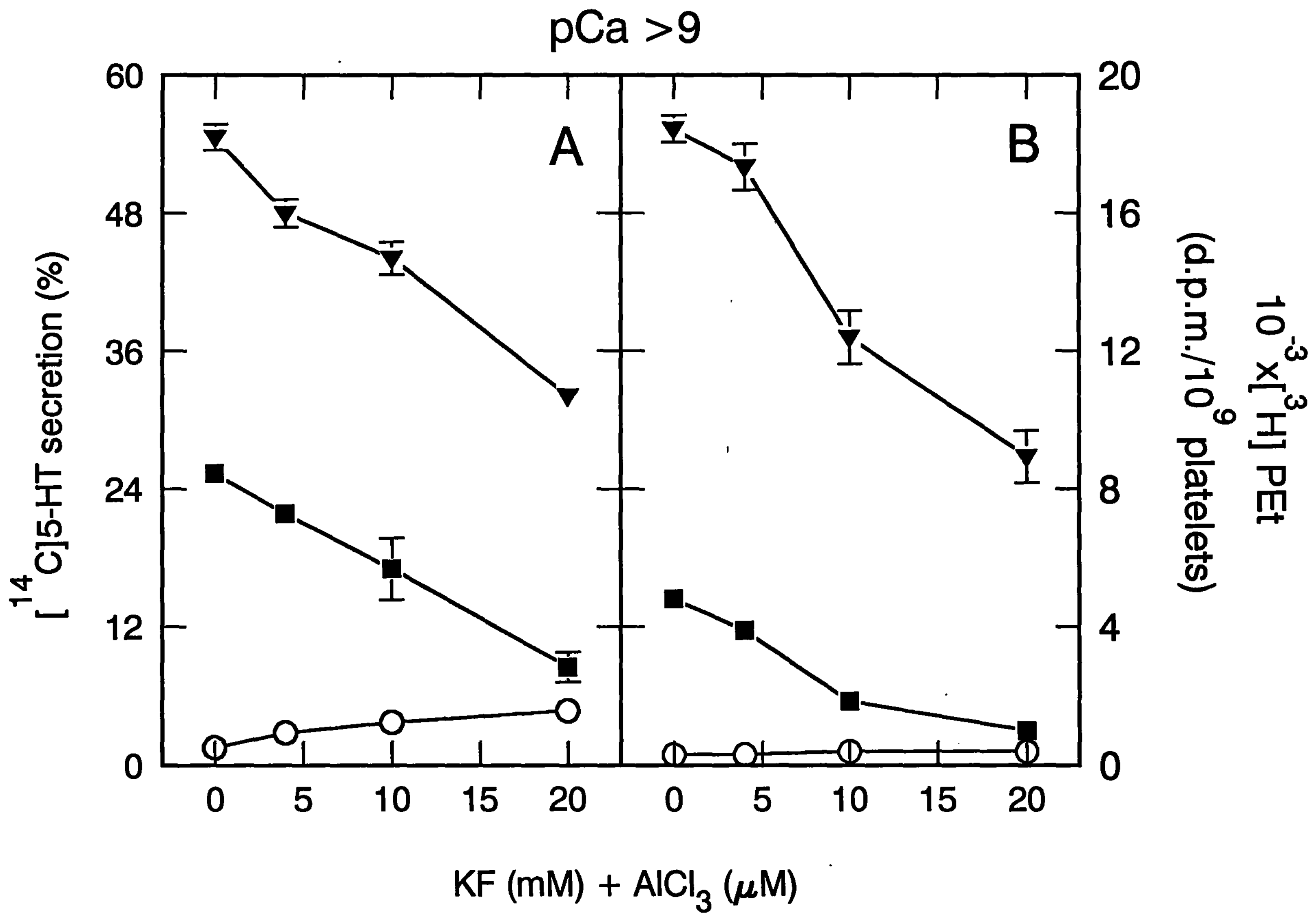
As shown in Fig. 6, in the absence of Ca^{2+} (pCa >9), KF/$AlCl_3$ did not induce [^{3}H]PEt formation or [^{14}C]5-HT secretion, but it did inhibit secretion and PLD activity induced by GTPγS in a concentration-dependent manner. With 10 mM KF and 10 μM $AlCl_3$, the inhibition of GTPγS-stimulated [^{3}H]PEt formation and [^{14}C]5-HT secretion amounted to 64 ± 4% (mean ± S.E., 3 expts.) and 41 ± 6% (mean ± S.E., 3 expts.), respectively. In addition, these concentrations of KF/$AlCl_3$

inhibited the $[^3H]$PEt formation and $[^{14}C]$5-HT secretion stimulated synergistically by GTPγS with PMA by 40 ± 6% (mean ± range, 2 expts.) and 27 ± 7% (mean ± range, 2 expts.), respectively. Thus, at pCa >9, the inhibitory effects of KF/$AlCl_3$ on GTPγS- or GTPγS plus PMA-induced $[^3H]$PEt formation (Fig. 6B) correlated with the inhibition of the $[^{14}C]$5-HT secretion (Fig. 6A).

3.3.2. Effects of KF/$AlCl_3$ on PLD activity at pCa 6. Even at a higher Ca^{2+} concentration (pCa 6), KF/$AlCl_3$ induced no additional formation of $[^3H]$PEt and $[^3H]$PA (Fig. 7B and C), suggesting it did not cause PLD activation. Although some $[^3H]$PA accumulated, it was not affected by the increased KF/$AlCl_3$ concentration, suggesting that this $[^3H]$PA was formed through other mechanisms. Ethanol inhibited both basal and KF/$AlCl_3$-induced secretion (Fig. 7A) and markedly decreased $[^3H]$PA formation (Fig. 7C). It follows that the inhibition of secretion by ethanol could not be attributed to an inhibition of a PLD activity induced by KF/$AlCl_3$, though it could be related to inhibition of $[^3H]$PA formation. For comparison, the concentration-dependent effects of GTPγS on $[^3H]$PEt and $[^3H]$PA accumulation and on $[^{14}C]$5-HT secretion are shown in Fig. 7 D, E and F. Ethanol inhibited GTPγS-induced secretion only with the lower GTPγS concentrations. In the presence of ethanol, the formation of $[^3H]$PEt was associated with decreased $[^3H]$PA accumulation caused by the ethanol. Interestingly, GTPγS at low concentrations (1-2 μM) that stimulate secretion similarly to KF/$AlCl_3$ caused only small increases in $[^3H]$PEt formation, but KF/$AlCl_3$ showed inhibitory effects on basal PLD activities. The effects of different

Figure 6. Effects of different concentrations of KF/$AlCl_3$ on secretion and [^{3}H]PEt formation stimulated by GTPγS or GTPγS and PMA in permeabilized platelets incubated at pCa >9

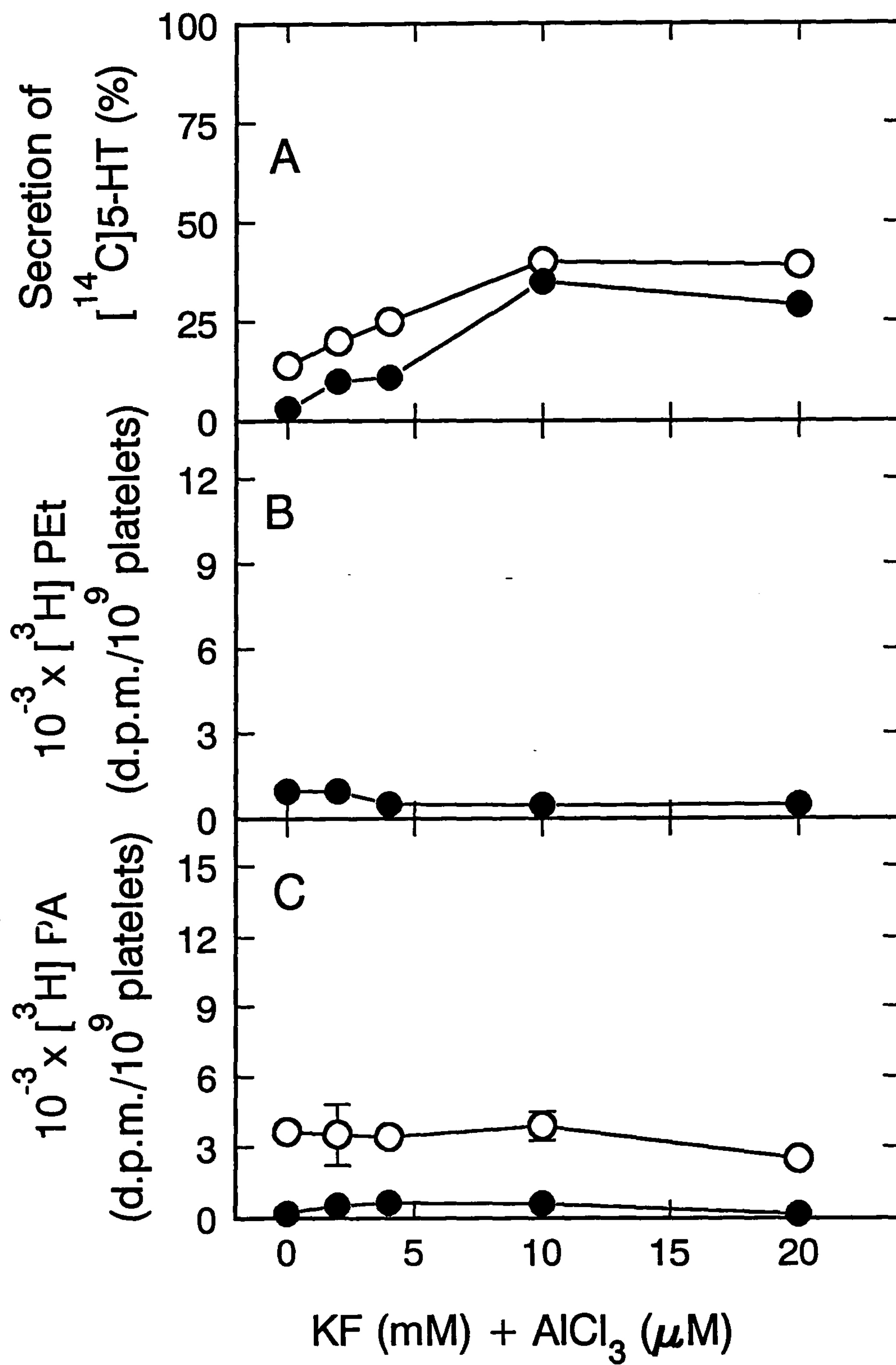
Samples of permeabilized platelets containing [^{14}C]5-HT and phospholipids labelled with [^{3}H]arachidonate were equilibrated (15 min at 0°C) and incubated (10 min at 25°C) with the indicated concentrations of KF + $AlCl_3$, and either no other addition (O), 100 μM GTPγS (■), or 100 μM GTPγS + 100 nM PMA (▼), all in the absence of Ca^{2+} (pCa>9) and in the presence of 200 mM ethanol. Secretion of [^{14}C]5-HT (A) and the formation of [^{3}H]PEt (B) were determined; values are means ± S.E. from triplicate samples. These results are from the same experiment as Fig. 4.



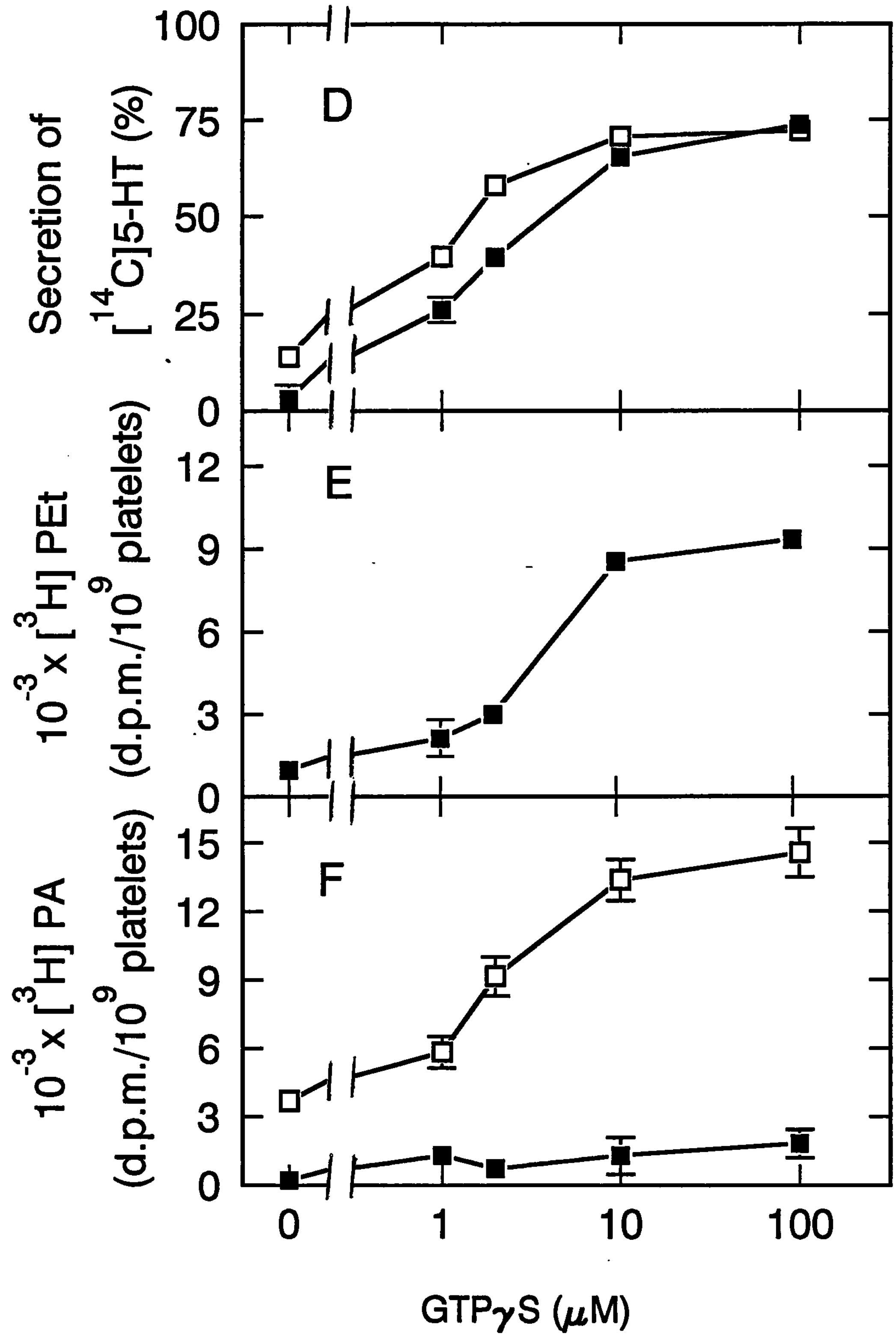
pCa >9
A
B
[14C]5-HT secretion (%)
60
48
36
24
12
0
10-3x[3H] PEt
(d.p.m./109 platelets)
20
16
12
8
4
0
0
5
10
15
20
KF (mM) + AlCl3 (μM)

Figure 7. Effects of different concentrations of KF/$AlCl_3$ or GTPγS on secretion and on the formation of phospholipid metabolites in permeabilized platelets; role of PLD

Samples of permeabilized platelets containing [^{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) at pCa 6, with the indicated concentrations of KF + $AlCl_3$ (A, B, C) or GTPγS (D, E, F), in the absence (open symbols) or presence (solid symbols) of 200 mM ethanol. Secretion of [^{14}C]5-HT (A, D) and the formation of [^{3}H]PEt (B, E) and [^{3}H]PA (C, F) were determined; values are means ± S.E. from triplicate samples.



Secretion of
[^{14}C]5-HT (%)
A
B
C
10^{-3} x [^{3}H] PEt
(d.p.m./10^9 platelets)
10^{-3} x [^{3}H] PA
(d.p.m./10^9 platelets)
100
75
50
25
0
12
9
6
3
0
15
12
9
6
3
0
0
5
10
15
20
KF (mM) + $AlCl_3$ (μM)

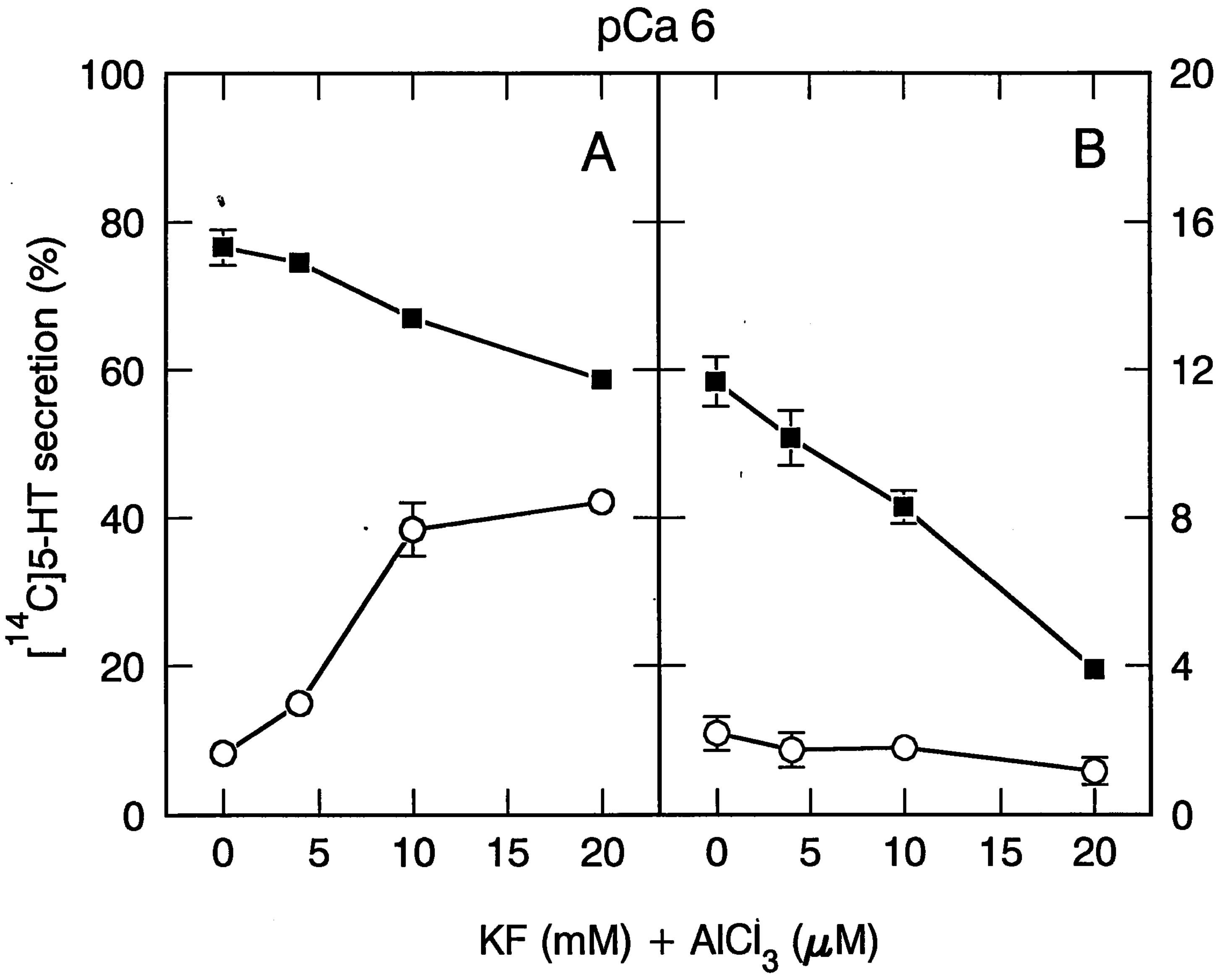

D
E
F
Secretion of [^{14}C]5-HT (%)
10^{-3} x [^{3}H] PEt (d.p.m./10^{9} platelets)
10^{-3} x [^{3}H] PA (d.p.m./10^{9} platelets)
100
75
50
25
0
12
9
6
3
0
15
12
9
6
3
0
0
1
10
100
GTPγS (μM)

concentrations of $KF/AlCl_3$ on GTPγS-induced secretion and [^{3}H]PEt formation at pCa 6 are shown in Fig. 8. Addition of 10 mM KF plus 10 μM $AlCl_3$ inhibited GTPγS-stimulated [^{3}H]PEt formation by 35 ± 6% (mean ± range, 2 expts.) and secretion by 8 ± 4% (mean ± range, 2 expts.). With 20 mM KF and 20 μM $AlCl_3$ the inhibition of GTPγS-stimulated [^{3}H]PEt formation and secretion were 67% and 23%, respectively. The inhibitory effects of $KF/AlCl_3$ on GTPγS-induced [^{3}H]PEt formation did not correlate well with the inhibition of secretion. To study this question further, 10 mM KF plus 10 μM $AlCl_3$ was added to permeabilized platelets incubated with different concentrations of GTPγS. Fig. 9 shows that $KF/AlCl_3$ increased secretion in the presence of a low concentration of GTPγS (2 μM) but slightly inhibited secretion induced by a high concentration of GTPγS (100 μM). In contrast, $KF/AlCl_3$ did not increase [^{3}H]PEt formation with any concentration of GTPγS and was markedly inhibitory with 10-100 μM GTPγS. These results again show a lack of correlation between secretion and [^{3}H]PEt formation at pCa 6.

3.3.3. Comparison of the effects of $KF/AlCl_3$, GTPγS and GTP on secretion and PLD activity. The results shown in Table 4 compare the effects of $KF/AlCl_3$ (10 mM and 10 μM, respectively), 1 μM GTPγS and 200 μM GTP on secretion and PLD activity at pCa 6. These three reagents caused similar extents of [^{14}C]5-HT secretion (about 40% in the absence of ethanol and 20% in the presence of ethanol). No significant increases in [^{3}H]PA and [^{3}H]PEt caused by these compounds were observed. Almost identical results were obtained in additional experiments in which

Figure 8. Effects of different concentrations of KF/$AlCl_3$ on GTPγS-induced secretion and [^{3}H]PEt formation in permeabilized platelets at pCa 6

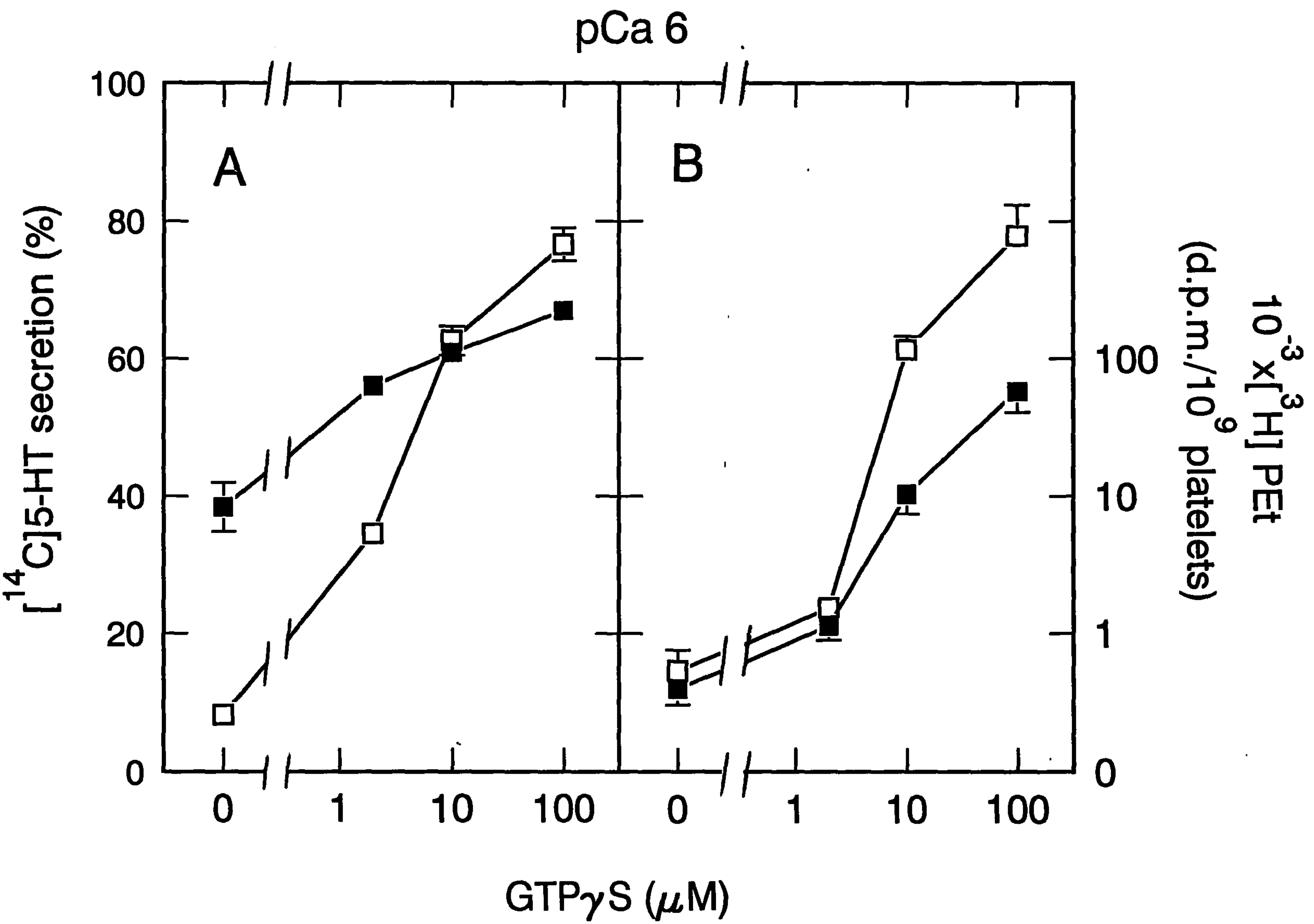
Samples of permeabilized platelets containing [^{14}C]5-HT and phospholipids labelled with [^{3}H]arachidonate were equilibrated (15 min at 0° C) and incubated (10 min at 25° C) with the indicated concentrations of KF + $AlCl_3$, and either no other addition (O), or 100 μM GTPγS (■), all at pCa 6 in the presence of 200 mM ethanol. Secretion of [^{14}C]5-HT (A) and the formation of [^{3}H]PEt (B) were determined; values are means ± S.E. from triplicate samples. These results are from the same experiment as Fig. 5.



pCa 6
A
B
[14C]5-HT secretion (%)
10-3x[3H] PEt
(d.p.m./10 9 platelets)
KF (mM) + AlCl3 (μM)
0
20
40
60
80
100
0
4
8
12
16
20
0
5
10
15
20

Figure 9. Effects of $KF/AlCl_3$ on secretion and $[^3H]$PEt formation induced by different concentrations of GTPγS in permeabilized platelets at pCa 6

Samples of permeabilized platelets containing $[^{14}C]$5-HT and phospholipids labelled with $[^3H]$arachidonate were equilibrated (15 min at 0°C) and incubated (10 min at 25°C) with the indicated concentrations of GTPγS, in the absence (□) or presence (■) of 10 mM KF + 10 μM $AlCl_3$, all at pCa 6 and with 200 mM ethanol. Secretion of $[^{14}C]$5-HT (A) and the formation of $[^3H]$PEt (B) were determined; values are means ± S.E. from triplicate samples.



pCa 6
A
B
[14C]5-HT secretion (%)
100
80
60
40
20
0
10-3x[3H] PEt
(d.p.m./109 platelets)
100
10
1
0
GTPγS (μM)
0
1
10
100

$KF/AlCl_3$ and GTPγS (2 μM) increased the secretion by 422 ± 42% and 522 ± 104% respectively (means ± S.E., 3 expts), whereas the basal [^{3}H]PEt formation was slightly decreased by $KF/AlCl_3$ (by 32 ± 9%) and slightly increased by 2 μM GTPγS (by 116 ± 45%) (means ± S.E., 3 expts). The difference between the effects of $KF/AlCl_3$ and GTPγS on [^{3}H]PEt formation was significant in a two-sided paried *t*-test ($P < 0.02$), though there was no significant difference on their effects on [^{14}C]5-HT secretion. These results indicate that $KF/AlCl_3$ and 1-2 μM GTPγS have similar effects on the stimulation of [^{14}C]5-HT secretion, but that their effects on PLD activity are different.

3.4. Effects of $KF/AlCl_3$ on phospholipase C (PLC) activity

3.4.1. Effects of $KF/AlCl_3$ on [^{3}H]DAG formation. As indicated in the above experiments, only in the absence of Ca^{2+} (pCa > 9), did PLD activity correlate well with secretion. In the presence of Ca^{2+} (pCa 6), PLD activity alone cannot mediate secretion. Therefore, the effects of $KF/AlCl_3$ on [^{3}H]DAG formation and PLC activity and on their relationship with secretion were examined.

With the addition of Ca^{2+} ions, $KF/AlCl_3$ markedly stimulated [^{3}H]DAG formation, as indicated in Fig. 10B. Addition of 10 mM KF with 10 μM $AlCl_3$ caused a 3.9 ± 0.6-fold increase in [^{3}H]DAG formation at pCa 6 (mean ± S.E., 5 expts.). In the absence of Ca^{2+}, no [^{3}H]DAG formation was detected (Fig. 10B), indicating that there is no PLC activation. The stimulation by $KF/AlCl_3$ of the secretion of

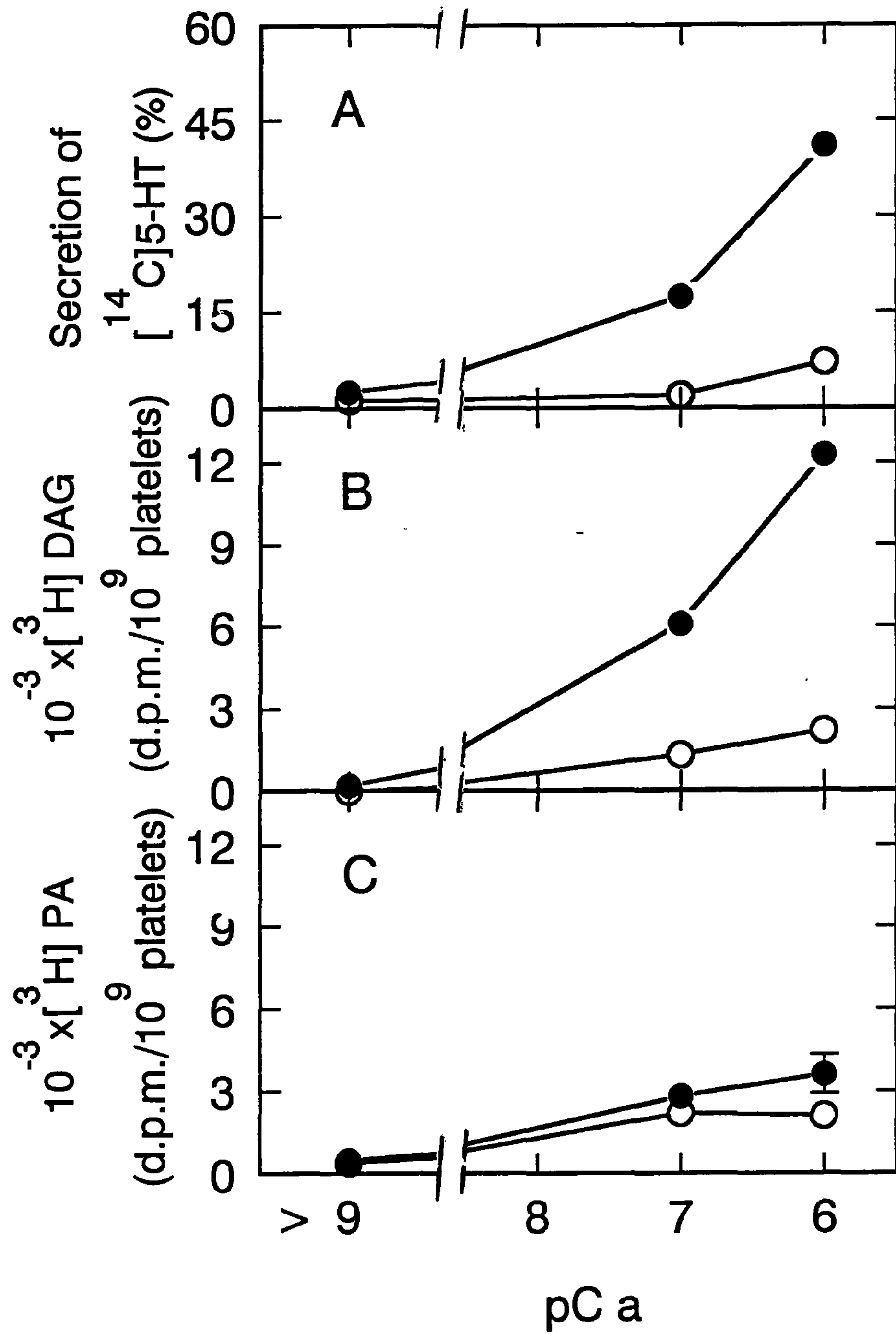
Table 4. Effects of $KF/AlCl_3$, GTPγS and GTP on secretion and the formation of phospholipid metabolites in permeabilized platelets

Samples of permeabilized platelets containing $[^{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) at pCa 6, with no stimuli, KF(10 mM) + $AlCl_3$ (10 μM), GTPγS (1 μM) or GTP (200 μM) in the absence or presence of ethanol (200 mM). Secretion of $[^{14}C]$5-HT and the formation of $[^{3}H]$PEt and $[^{3}H]$PA were determined; values are means ± S.E. from triplicate samples.

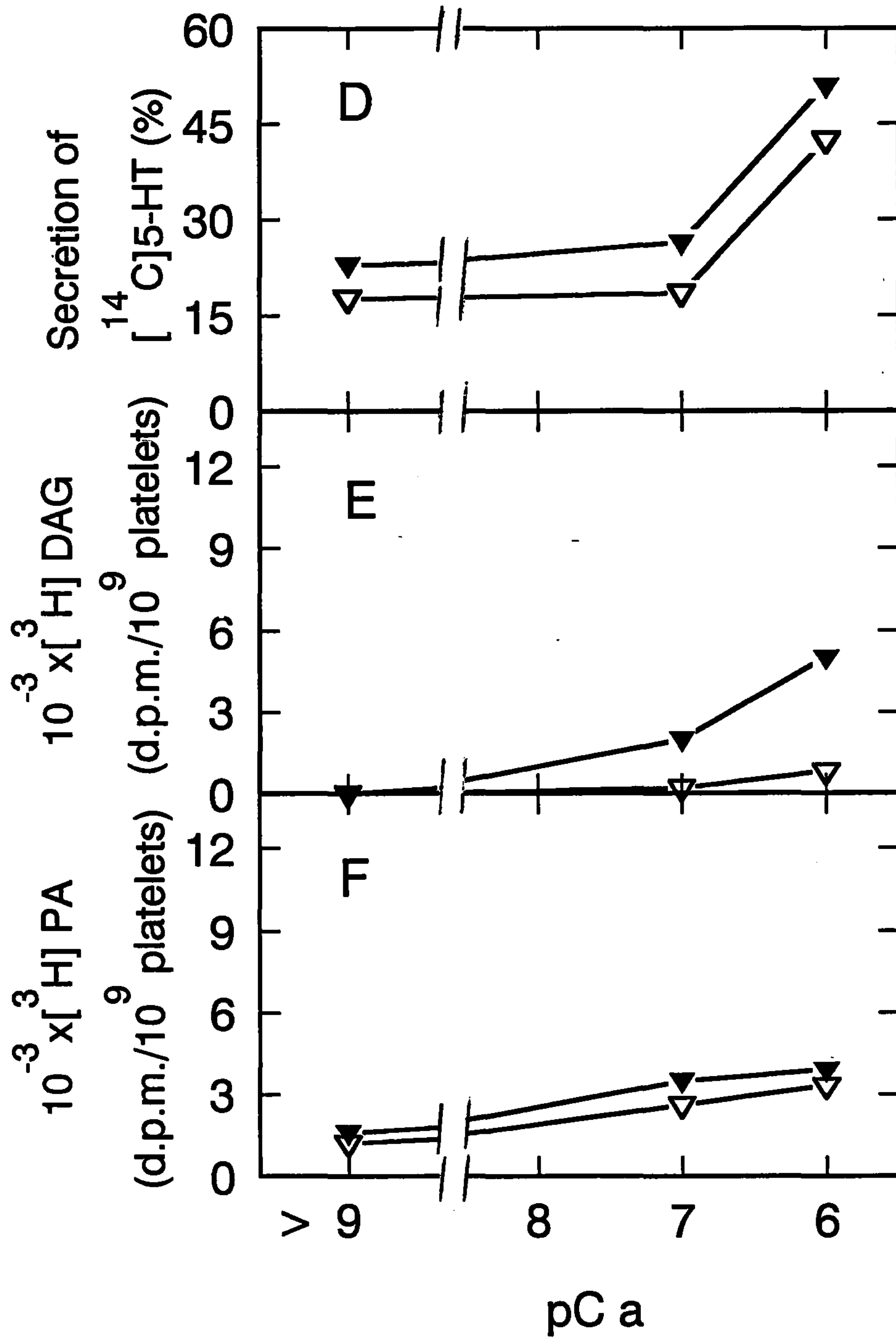
Additions		Secretion of [^{14}C]5-HT	Formation of [^{3}H]phospholipid metabolites (10^{-3}x d.p.m./10^{9} platelets)	
Stimuli	Ethanol	(%)	[^{3}H]PA	[^{3}H]PEt
None	-	12 ± 2	6.2 ± 0.4	---
	+	5 ± 1	4.8 ± 0.2	1.2 ± 0.3
KF + $AlCl_3$	-	41 ± 2	7.6 ± 0.8	---
	+	25 ± 1	5.0 ± 1.1	0.8 ± 0.4
GTPγS	-	36 ± 3	7.7 ± 0.5	---
	+	22 ± 2	6.8 ± 0.5	1.3 ± 0.4
GTP	-	45 ± 1	7.1 ± 0.5	---
	+	20 ± 0	5.6 ± 0.7	0.9 ± 0.2

Figure 10. Effects of $KF/AlCl_3$ on secretion and on the formation of phospholipid metabolites in permeabilized platelets at various buffered Ca^{2+} concentrations

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 then incubated (10 min at 25°C) with the indicated Ca^{2+} buffers in the absence (open symbols) or presence (solid symbols) of 10 mM KF + 10 μM $AlCl_3$, with either no other addition (A-C) or 100 nM PMA (D-F). No ethanol was present. Secretion of [^{14}C]5-HT (A, D) and the formation of [^{3}H]DAG (B, E) and [^{3}H]PA (C, F) were determined; values are means ± S.E. from triplicate samples.



A
B
C
Secretion of
[14C]5-HT (%)
10-3 x[3H] DAG
(d.p.m./10 9 platelets)
10-3 x[3H] PA
(d.p.m./10 9 platelets)
60
45
30
15
0
12
9
6
3
0
12
9
6
3
0
> 9
8
7
6
pC a


D
E
F
Secretion of
[14C]5-HT (%)
10-3 x [3H] DAG
(d.p.m./10^9 platelets)
10-3 x [3H] PA
(d.p.m./10^9 platelets)
60
45
30
15
0
12
9
6
3
0
12
9
6
3
0
> 9
8
7
6
pCa

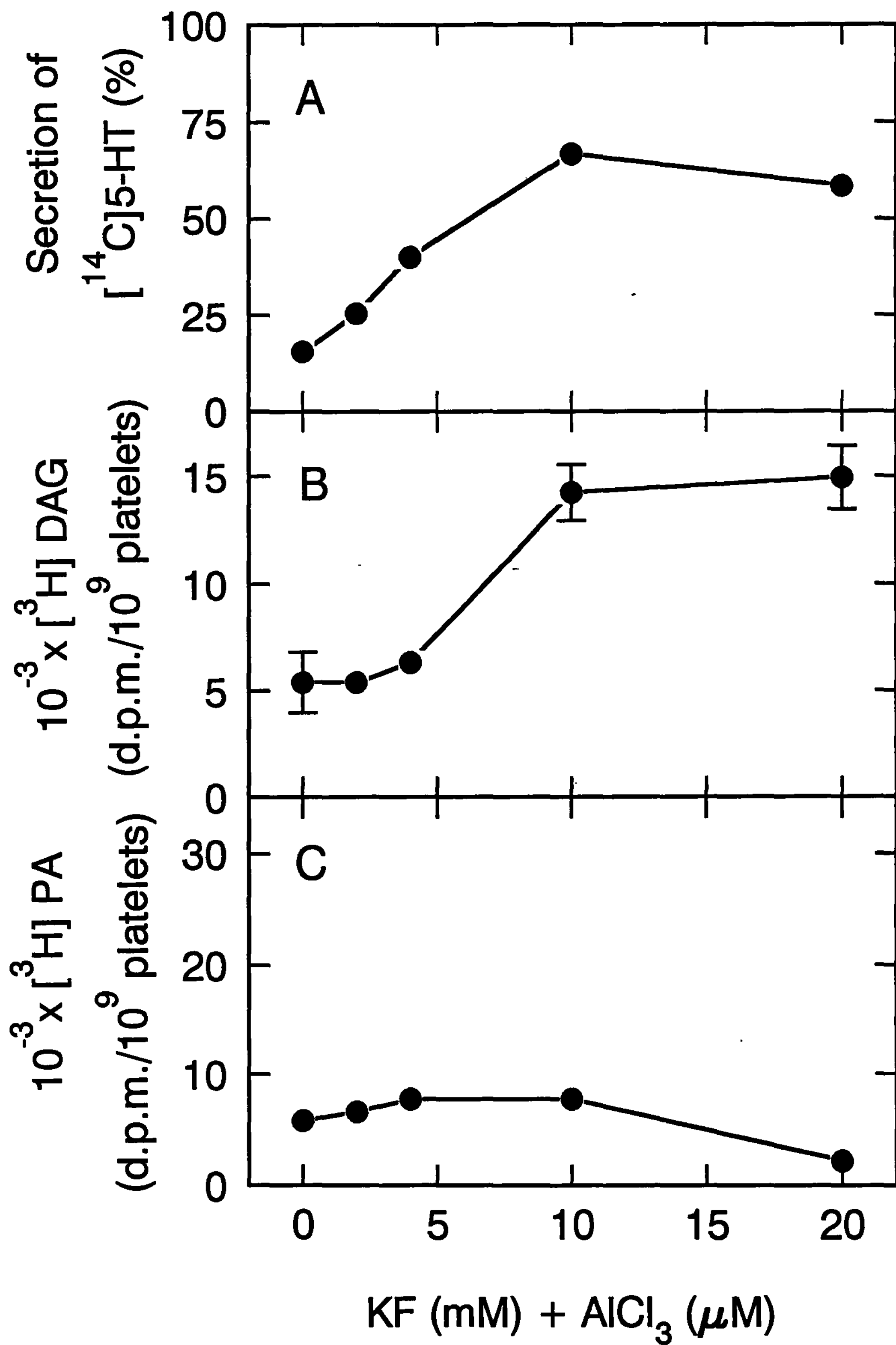
[^{14}C]5-HT correlated better with its effect on the accumulation of [^{3}H]DAG (Fig. 10 A and B) than with its action on the accumulation of [^{3}H]PA (Fig. 10 C). The concentration dependence of the effect of KF/$AlCl_3$ on [^{3}H]DAG accumulation is shown in Fig. 11B, and correlated roughly with [^{14}C]5-HT secretion, which is shown in Fig. 11A. The lack of correlation between [^{3}H]PA formation and [^{14}C]5-HT secretion was observed again (Fig. 11 A and C).

Although PMA had a small stimulatory effect on KF/$AlCl_3$-induced secretion (Fig.10D), this compound inhibited KF/$AlCl_3$-induced [^{3}H]DAG formation (Fig. 10E). PMA also inhibited basal [^{3}H]DAG formation. It has previously been found that PMA inhibits DAG generation in this experimental system through an inhibition of phospholipase C (Coorssen *et al.*, 1990). This suggests that KF/$AlCl_3$ stimulates DAG formation by activating phospholipase C. PMA also caused small increases in [^{3}H]PA formation (Fig. 10F), which may have been generated by PLD activation.

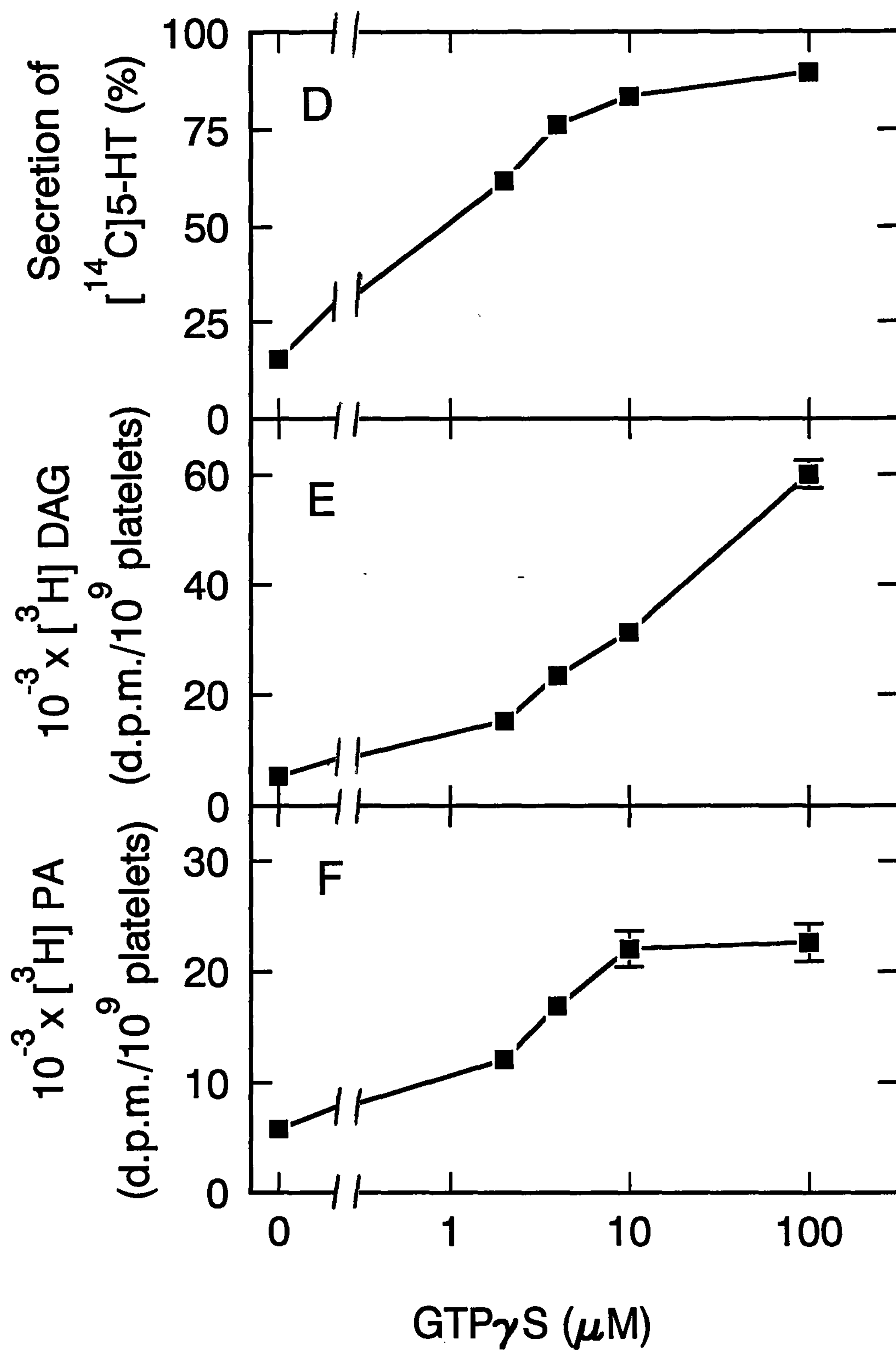
3.4.2. Effects of KF/$AlCl_3$ on GTPγS-induced [^{3}H]DAG formation. Relative to the effects of KF/$AlCl_3$ (Fig. 11A, B and C), increasing concentrations of GTPγS much more markedly stimulated the formation of both [^{3}H]DAG and [^{3}H]PA, as well as [^{14}C]5-HT secretion (Fig. 11D, E and F). Addition of 2 μM, 4 μM, 10 μM, and 100 μM GTPγS caused 2.8-, 4.4-, 5.8- and 11.2-fold increases in [^{3}H]DAG accumulation, respectively, and also caused 2.1-, 2.9-, 3.8- and 3.9-fold increases in [^{3}H]PA, respectively. These results together with the previous studies suggest that GTPγS activated both PLC and PLD in the presence of Ca^{2+}. However, KF/$AlCl_3$ was only

Figure 11. Effects of different concentrations of $KF/AlCl_3$ and GTPγS on secretion and on the formation of phospholipid metabolites in permeabilized platelets; role of PLC

Samples of permeabilized platelets containing [^{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) at pCa 6 with the indicated concentrations of KF + $AlCl_3$ (●) or GTPγS (■), all in the absence of ethanol. Secretion of [^{14}C]5-HT (A, D) and the formation of [^{3}H]DAG (B, E) and [^{3}H]PA (C, F) were determined; values are means ± S.E. from triplicate samples.



A
B
C
Secretion of [14C]5-HT (%)
10-3 x [3H] DAG (d.p.m./109 platelets)
10-3 x [3H] PA (d.p.m./109 platelets)
100
75
50
25
0
15
10
5
0
30
20
10
0
0
5
10
15
20
KF (mM) + AlCl3 (μM)



Secretion of
[14C]5-HT (%)
100
75
50
25
0
D
10^{-3} x [3H] DAG
(d.p.m./10^9 platelets)
60
40
20
0
E
10^{-3} x [3H] PA
(d.p.m./10^9 platelets)
30
20
10
0
F
0
1
10
100
GTPγS (μM)

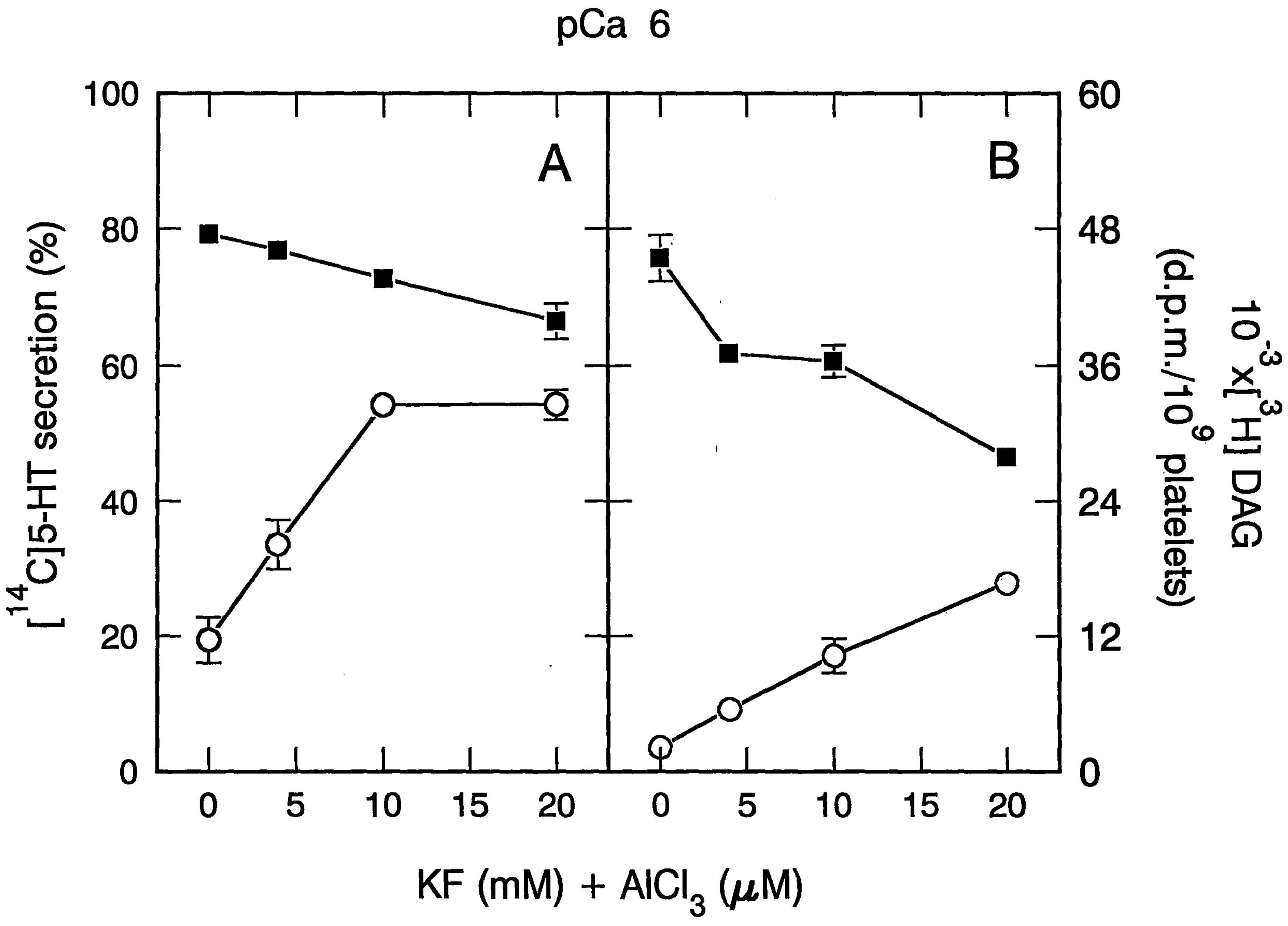
able to activate PLC in these experiments.

When platelets were incubated with different concentrations of KF/$AlCl_3$ and 100 μM GTPγS, as shown in Fig. 12, KF/$AlCl_3$ slightly inhibited both secretion and [^{3}H]DAG formation stimulated by GTPγS. Addition of 10 mM KF with 10 μM $AlCl_3$ decreased GTPγS-induced [^{14}C]5-HT secretion and [^{3}H]DAG accumulation by 6 ± 2% (mean ± range, 2 expts.) and 19 ± 1% (mean ± range, 2 expts.), respectively. A higher concentration of 20 mM KF with 20 μM $AlCl_3$ caused a 16% decrease in secretion and a 38% decrease in [^{3}H]DAG formation. As shown in Fig. 13, 10 mM KF with 10 μM $AlCl_3$ increased both the secretion and [^{3}H]DAG accumulation caused by low concentrations of GTPγS, but decreased the effects of 100 μM GTPγS. Both Fig. 12 and Fig. 13 indicate that, at pCa 6, the effects of KF/$AlCl_3$ on GTPγS-induced [^{3}H]DAG formation correlated well with their corresponding effects on [^{14}C]5-HT secretion.

3.4.3. Effects of KF/$AlCl_3$ on [^{3}H]inositol phosphate formation. To obtain evidence that the KF/$AlCl_3$-induced formation of DAG was due to the activation of phosphoinositide-specific phospholipase C, the release of [^{3}H]inositol phosphates was measured in platelets labelled with [^{3}H]inositol before permeabilization and incubation with KF/$AlCl_3$. As shown in Table 5, at pCa 6 in the absence of Li^+, 10 mM KF with 10 μM $AlCl_3$ increased [^{3}H]IP accumulation to a significant extent ($P < 0.02$). Addition of 1 μM GTPγS had a similar effect on the formation of [^{3}H]IP. In the presence of Li^+, an inhibitor of both IP and IP_2 phosphatases, 10 mM KF with

Figure 12. Effects of different concentrations of $KF/AlCl_3$ on GTPγS induced secretion and [^{3}H]DAG formation in permeabilized platelets at pCa 6

Samples of permeabilized platelets containing [^{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 concentrations of KF + $AlCl_3$, and either no other addition (O), or 100 μM GTPγS (■), all at pCa 6 in the absence of ethanol. Secretion of [^{14}C]5-HT (A) and the formation of [^{3}H]DAG (B) were determined; values are means ± S.E. from triplicate samples. These results are from the same experiment as Fig. 5.



pCa 6
A
B
[14C]5-HT secretion (%)
100
80
60
40
20
0
60
48
36
24
12
0
10-3x[3H] DAG
(d.p.m./109 platelets)
0
5
10
15
20
KF (mM) + AlCl3 (μM)

Figure 13. Effects of KF/$AlCl_3$ on secretion and [^{3}H]DAG formation induced by different concentrations of GTPγS in permeabilized platelets at pCa 6

Samples of permeabilized platelets containing [^{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 concentrations of GTPγS, in the absence (□) or presence (■) of 10 mM KF + 10 μM $AlCl_3$, all at pCa 6 and without ethanol. Secretion of [^{14}C]5-HT (A) and the formation of [^{3}H]DAG (B) were determined; values are means ± S.E. from triplicate samples. These results are from the same experiment as Fig. 9.

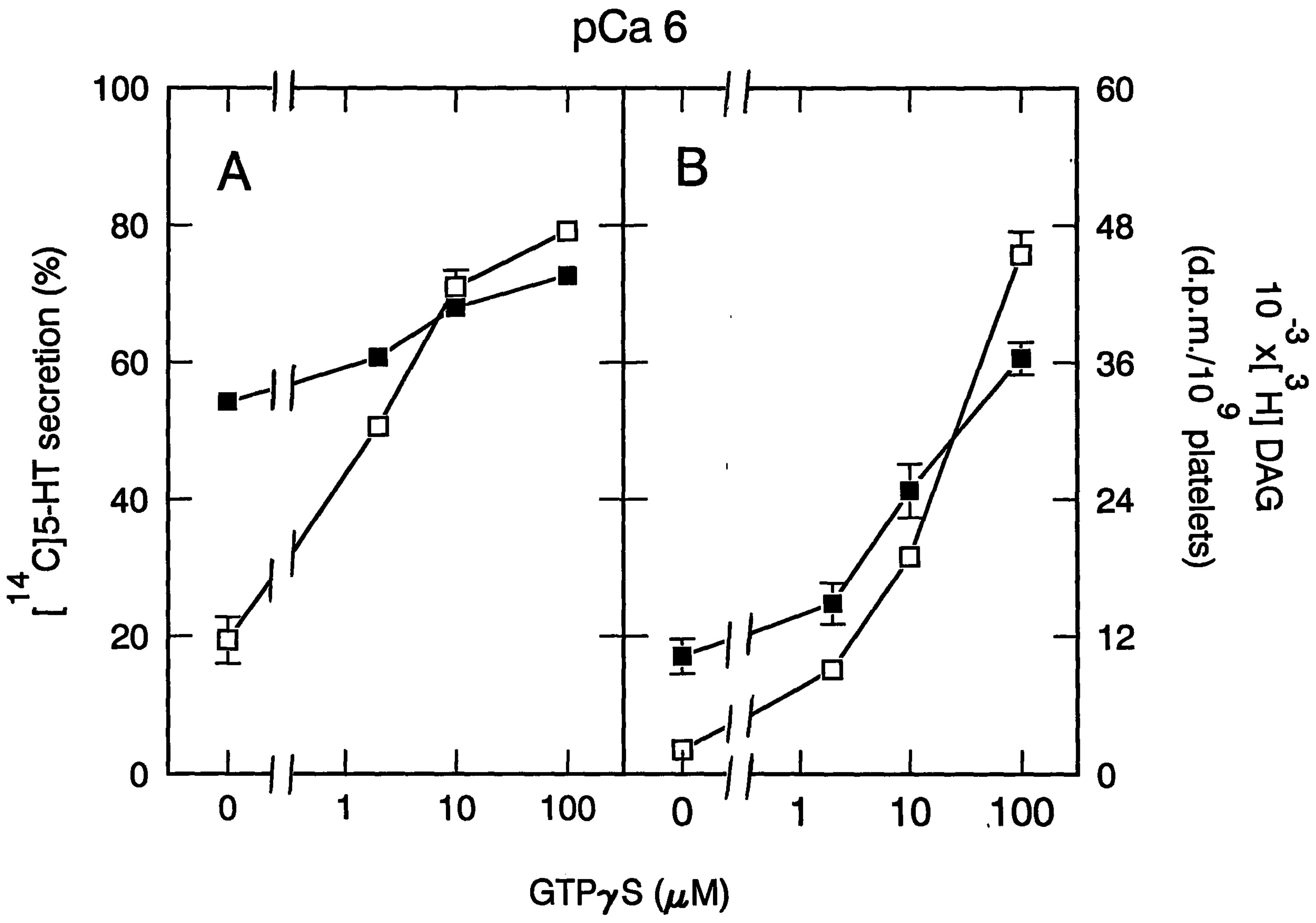

pCa 6
A
B
[14C]5-HT secretion (%)
10-3 x[3H] DAG
(d.p.m./109 platelets)
GTPγS (μM)
0
1
10
100
0
20
40
60
80
100
0
12
24
36
48
60

Table 5. The effects of $KF/AlCl_3$ on the formation of [^{3}H]inositol phosphates in permeabilized platelets

Platelets labelled with [^{3}H]inositol in an $MnCl_2$/low-glucose medium were permeabilized. Samples were equilibrated (15 min at 0°C) and incubated (10 min at 25°C) at pCa 6 with the indicated additions, in the presence or absence of LiCl (10 mM), before extraction and measurement of [^{3}H]inositol phosphates. Values are means ± S.E. from triplicate samples. The significance of changes was evaluated by two-sided unpaired *t*-tests ($^*P < 0.02$).

	Additions	^{3}H present in inositol phosphates (dpm/10^{9} platelets)		
		IP	IP_2	IP_3
- LiCl	None	270 ± 54	338 ± 52	54 ± 28
	KF (10 mM) + $AlCl_3$ (10 μM)	598 ± 58*	419 ± 37	30 ± 17
	GTPγS (1 μM)	539 ± 61*	539 ± 42	69 ± 14
+ LiCl	None	359 ± 70	222 ± 37	0 ± 21
	KF (10 mM) + $AlCl_3$ (10 μM)	388 ± 45	517 ± 41*	8 ± 10
	KF (20 mM) + $AlCl_3$ (20 μM)	514 ± 58	673 ± 40*	13 ± 43
	GTPγS (1 μM)	622 ± 66	447 ± 69*	41 ± 27
	GTPγS (100 μM)	3027 ± 153*	2459 ± 107*	112 ± 24*
	GTPγS(100 μM) + KF(10 mM) + $AlCl_3$(10 μM)	1740 ± 44*	2678 ± 75*	75 ± 23

10 μM $AlCl_3$ increased $[^3H]IP_2$ accumulation (by 2.3-fold) rather than that of $[^3H]IP$, though the total formation of $[^3H]IP$ and $[^3H]IP_2$ was the same as in the absence of Li^+. This indicates that in this experiment, Li^+ acted mainly by inhibiting the breakdown of IP_2. Addition of 100 μM GTPγS increased the total formation of $[^3H]IP$ and $[^3H]IP_2$ by 9.4-fold and this effect was inhibited by 19% by KF (10 mM) with $AlCl_3$ (10 μM). The effect of 100 μM GTPγS was 5-fold greater than that of 1 μM GTPγS with respect to the total formation of $[^3H]IP$ and $[^3H]IP_2$. These results are consistent with the effcts of KF/$AlCl_3$ and GTPγS on $[^3H]DAG$ accumulation, in which the effect of 100 μM GTPγS was 4-fold greater than that of 2 μM GTPγS, and KF (10 mM)/$AlCl_3$ (10 μM) inhibited the effect of 100 μM GTPγS by 19 ± 1%. IP_3 accumulation was only significant with 100 μM GTPγS.

Taken collectively, these results suggest that the DAG formation occurring in the presence of KF/$AlCl_3$ is likely to result from the activation of phosphoinositide-specific phospholipase C.

3.5. *Effects of vanadate/H_2O_2*

Since vanadate has been found to have similar effects to AlF_4^- in inducing cellular activation, it was of interest to examine its effects on $[^{14}C]$5-HT secretion in relation to PLD and PLC activation in permeabilized platelets.

3.5.1. Effects of vanadate/H_2O_2 on secretion. Table 6 shows that, in the absence of Ca^{2+} (pCa > 9), vanadate (200 μM) added either alone or with H_2O_2 (2 mM)

Table 6. Effects of vanadate/H_2O_2 on secretion and pleckstrin phosphorylation in permeabilized platelets

Samples of permeabilized platelets containing $[^{14}C]$5-HT and when required, $[\gamma\text{-}^{32}P]$ATP, were equilibrated (15 min at 0°C) and incubated (10 min at 25°C) with the indicated additions of vanadate (200 μM) or H_2O_2 (2 mM) at various buffered Ca^{2+} concentrations. Secretion of $[^{14}C]$5-HT (triplicate samples) and the phosphorylation of pleckstrin (duplicate samples) were determined; values are means ± S.E. or means ± range, respectively.

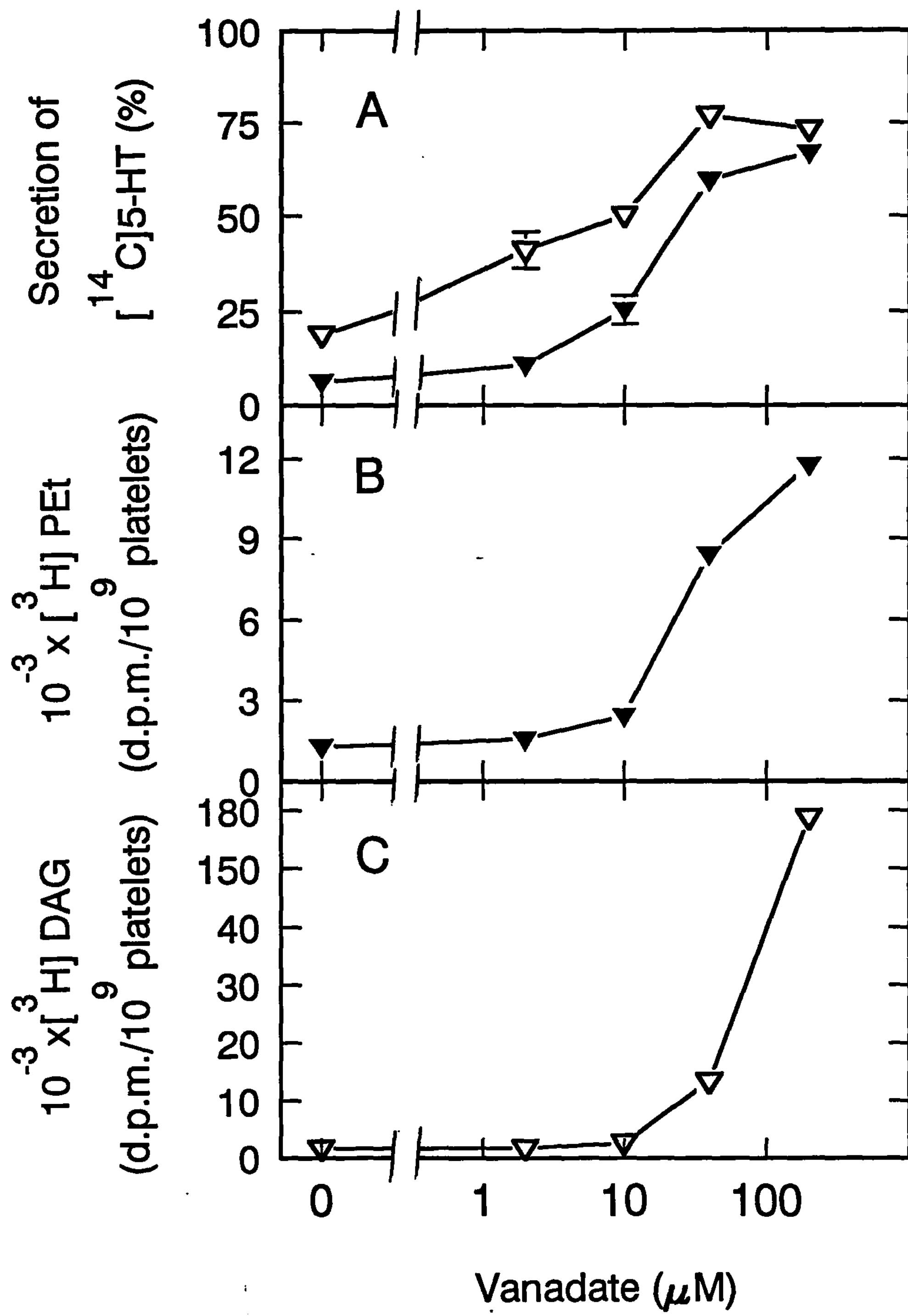
pCa	Additions	Secretion of $[^{14}C]$5-HT (%)	Phosphorylation of pleckstrin (pmol $^{32}PO_4/10^9$) platelets)
>9	None	4 ± 0	177 ± 10
	Vanadate	9 ± 0	624 ± 32
	H_2O_2	4 ± 1	291 ± 4
	Vanadate + H_2O_2	10 ± 1	703 ± 16
7.0	None	4 ± 1	268 ± 16
	Vanadate	18 ± 1	775 ± 4
	H_2O_2	9 ± 1	431 ± 2
	Vanadate + H_2O_2	14 ± 0	805 ± 111
5.5	None	25 ± 6	431 ± 27
	Vanadate	65 ± 2	616 ± 22
	H_2O_2	58 ± 4	471 ± 18
	Vanadate + H_2O_2	58 ± 2	670 ± 89
4.5	None	68 ± 2	416 ± 39
	Vanadate	64 ± 2	721 ± 18
	H_2O_2	73 ± 1	455 ± 31
	Vanadate + H_2O_2	68 ± 2	832 ± 44

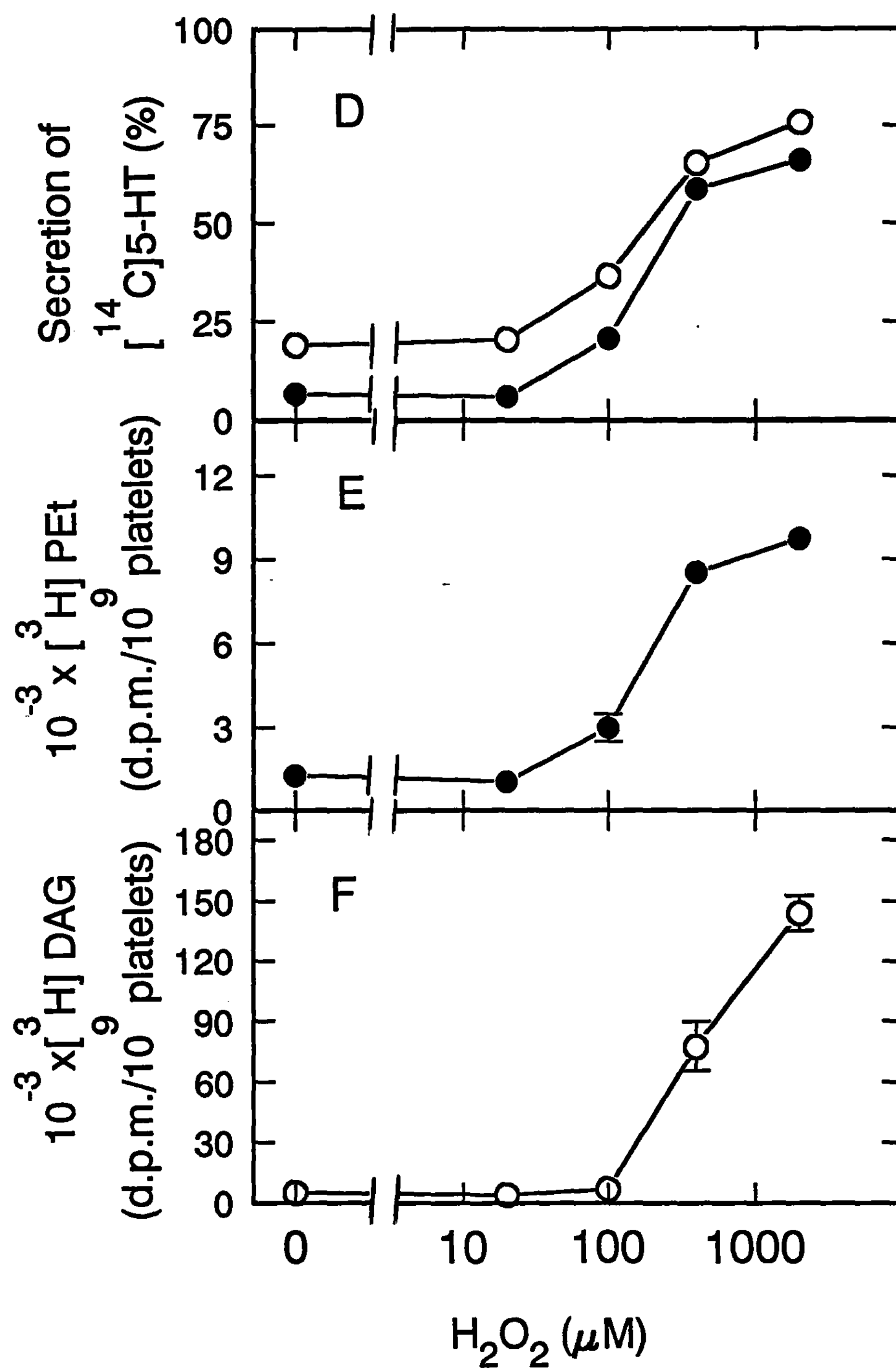
had only a very small effect on [^{14}C]5-HT secretion. In the presence of increasing Ca^{2+} concentrations, vanadate, H_2O_2 or both stimulated a Ca^{2+}-dependent [^{14}C]5-HT secretion. At pCa 5.5, 200 μM vanadate alone caused an almost maximal secretion of 65% of [^{14}C]5-HT and H_2O_2 had no additional effects on vanadate-induced secretion (Table 6). However, controls showed that H_2O_2 alone caused secretion. Experiments were then carried out using different concentrations of vanadate and/or H_2O_2 at pCa 6. As shown in Fig. 14A and D, either vanadate or H_2O_2 caused concentration-dependent secretion of [^{14}C]5-HT. A maximum secretion of 72 ± 5% of [^{14}C]5-HT (mean ± range, 2 expts.) was induced by 40 μM vanadate. Even 2 μM vanadate could induce a 41% secretion of [^{14}C]5-HT (Fig. 14A). Although 20 μM H_2O_2 had no effect, 100 μM H_2O_2 caused a 37% secretion and 2 mM H_2O_2 caused the maximum secretion of 73 ± 1% (mean ± S.E., 3 expts.) (Fig. 14D). When vanadate was added with 10-fold the concentration of H_2O_2, a synergistic effect of low concentrations of these stimuli was observed. Addition of 2 μM vanadate together with 20 μM H_2O_2, caused a maximal secretion of about 76% (Fig. 14G). Ethanol partly inhibited the secretion stimulated by vanadate or H_2O_2 alone, but had little effect on the secretion caused by concentrations of these stimuli that had maximal effects (Fig. 14A, D and G).

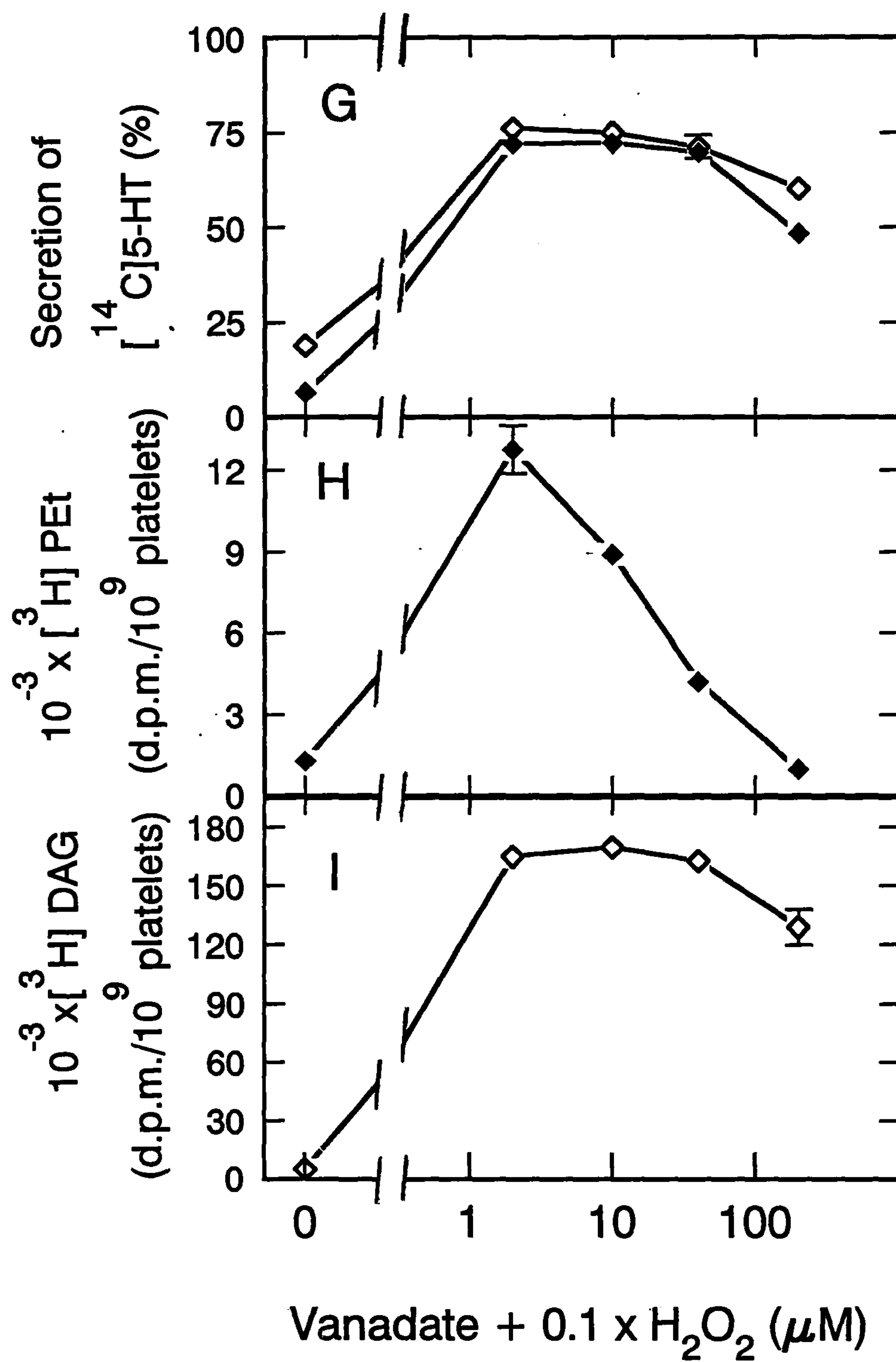
3.5.2. Effects of vanadate/H_2O_2 on protein phosphorylation. After incubating permeabilized platelets with vanadate, or vanadate and H_2O_2, ^{32}P incorporation from [γ-^{32}P]ATP into pleckstrin (P47) was observed, whether Ca^{2+} was present or not

Figure 14. Effects of different concentrations of vanadate and H_2O_2 on secretion and on the formation of phospholipid metabolites in permeabilized platelets

Samples of permeabilized platelets containing [^{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) at pCa 6, with the indicated concentrations of vanadate (A-C), or H_2O_2 (D-F), or vanadate + H_2O_2 (G-I), in the absence (open symbols) or presence (solid symbols) of 200 mM ethanol. Secretion of [^{14}C]5-HT (A, D and G) and the formation of [^{3}H]PEt (B, E and H) and [^{3}H]DAG (C, F and I) were determined; values are means ± S.E. from triplicate samples.


A
B
C
Secretion of
[^{14}C]5-HT (%)
10^{-3} x [^{3}H] PEt
(d.p.m./10^{9} platelets)
10^{-3} x[^{3}H] DAG
(d.p.m./10^{9} platelets)
Vanadate (μM)
0
1
10
100
25
50
75
3
6
9
12
10
20
30
40
150
180


D
E
F
Secretion of [14C]5-HT (%)
10-3 x [3H] PEt (d.p.m./109 platelets)
10-3 x[3H] DAG (d.p.m./109 platelets)
100
75
50
25
0
12
9
6
3
0
180
150
120
90
60
30
0
0
10
100
1000
H2O2 (μM)

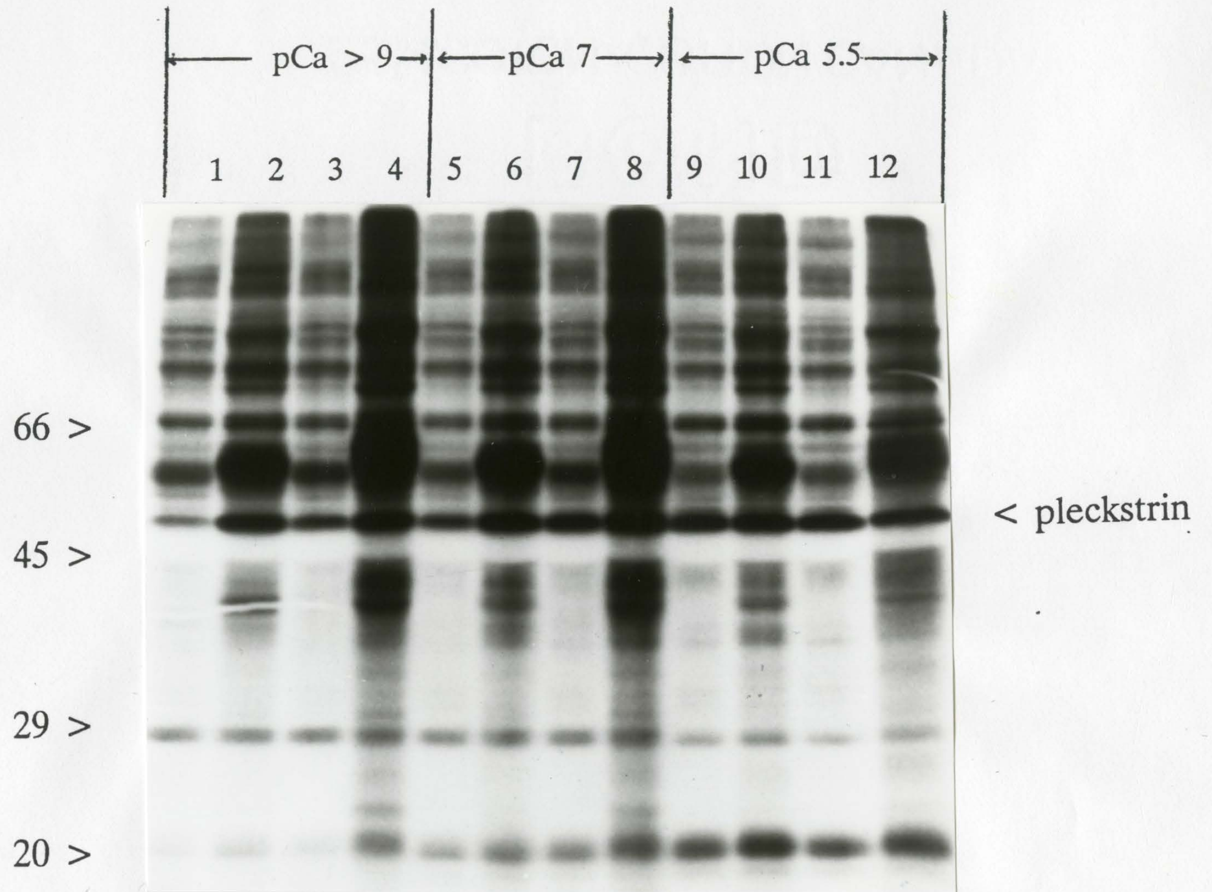

G
H
I
Secretion of [14C]5-HT (%)
10-3 x [3H] PEt (d.p.m./109 platelets)
10-3 x[3H] DAG (d.p.m./109 platelets)
100
75
50
25
0
12
9
6
3
0
180
150
120
90
60
30
0
0
1
10
100
Vanadate + 0.1 x H2O2 (μM)

(Table 6). H_2O_2 alone had small effects on the phosphorylation of pleckstrin at pCa >9 and 7 (Table 6). In addition, as shown in Fig. 15, either vanadate or vanadate with H_2O_2 increased ^{32}P incorporation into several other proteins which have been identified as protein-tyrosine kinase substrate in previous studies (Inazu *et al.*, 1990; Lerea *et al.*, 1989).

3.5.3. Effects of vanadate/H_2O_2 on PLD activity. The above results showed that vanadate stimulated a Ca^{2+}-dependent secretion, as did KF/$AlCl_3$, but that these two agents had different effects on protein phosphorylation. It was therefore of interest to examine the relationship of secretion to the activation of PLD and PLC in permeabilized platelets incubated with vanadate or H_2O_2. As shown in Fig. 14B, E and H, [^{3}H]PEt accumulation was measured at pCa 6 in the presence of 200 mM ethanol. Either vanadate or H_2O_2 stimulated the [^{3}H]PEt formation in a concentration-dependent manner. Addition of 200 µM vanadate and 2 mM H_2O_2 caused 9- and 7.5-fold increases in [^{3}H]PEt respectively, whereas 2 µM vanadate or 20 µM H_2O_2 had no effect on [^{3}H]PEt formation (Fig. 14B and E). When 2 µM vanadate was added together with 20 µM H_2O_2, these compounds acted synergistically to increase [^{3}H]PEt accumulation by more than 9-fold, as shown in Fig. 14H. In contrast, higher concentrations of vanadate plus H_2O_2 inhibited [^{3}H]PEt formation in a concentration-dependent manner. [^{3}H]PEt formation was completely abolished by 200 µM vanadate with 2 mM H_2O_2 (Fig. 14H), though each of these two agents increased [^{3}H]PEt to a maximal level alone (Fig. 14B and E). Although

Figure 15. Effects of vanadate/H_2O_2 on protein phosphorylation in permeabilized platelets at various buffered Ca^{2+} concentrations

Samples of permeabilized platelets containing [γ-^{32}P]ATP were equilibrated (15 min at 0°C) and incubated (10 min at 25°C) with Ca^{2+} buffers giving pCa values of > 9 (lane 1-4), 7 (lane 5-8) and 5.5 (lane 9-12). Other additions were as follows: lanes 1, 5 and 9, none; lanes 2, 6 and 10, vanadate (200 μM); lanes 3, 7 and 11, H_2O_2 (2 mM); lanes 4, 8, and 12, vanadate (200 μM) plus H_2O_2 (2 mM). Protein was then precipitated with trichloroacetic acid and resolved by SDS/polyacrylamide-gel electrophoresis; an autoradiograph of the dried gel is shown.


pCa > 9
pCa 7
pCa 5.5
1 2 3 4 5 6 7 8 9 10 11 12
66 >
45 >
29 >
20 >
< pleckstrin

the effects of vanadate or H_2O_2 on PLD activity correlated with their effects on secretion (Fig. 14, A and B, D and E), the effects of vanadate with H_2O_2 on PLD activity did not correlate well with secretion (Fig. 14G and H).

3.5.4. Effects of vanadate/H_2O_2 on [^{3}H]DAG formation. As shown in Fig. 14C, F and I, either vanadate or H_2O_2 increased [^{3}H]DAG formation in a concentration-dependent manner at pCa 6. Addition of 2 μM vanadate or 20 μM H_2O_2 had little effect on [^{3}H]DAG formation, but 200 μM vanadate or 2 mM H_2O_2 alone increased [^{3}H]DAG accumulation by more than 25-fold (Fig. 14C and F). As shown in Fig. 14I, low concentrations of vanadate and H_2O_2 exerted synergistic effects on [^{3}H]DAG accumulation, as was also observed for [^{3}H]PEt in the presence of ethanol. The maximum [^{3}H]DAG accumulation was obtained with 2 μM vanadate plus 20 μM H_2O_2. Higher concentrations of vanadate with H_2O_2 maintained [^{3}H]DAG accumulation at a level close to the maximum, in contrast with [^{3}H]PEt. Only 200 μM vanadate with 2 mM H_2O_2 decreased [^{3}H]DAG formation significantly relative to the maximum response. These synergistic effects of vanadate with H_2O_2 on [^{3}H]DAG accumulation correlated well with their effects on [^{14}C]5-HT secretion (Fig. 14G and I). This is consistent with the results observed in KF/$AlCl_3$-treated permeabilized platelets. Thus at pCa 6, DAG accumulation correlated much better with 5-HT secretion than did PEt formation.

3.5.5. The effects of vanadate/H_2O_2 on secretion and on [^3H]PEt and [^3H]DAG formation induced by GTPγS. At pCa 6 in the presence of ethanol, vanadate (200 µM) or vanadate (40 µM) with 2 mM H_2O_2 both slightly inhibited GTPγS-induced [^{14}C]5-HT secretion (by 12%) (Table 7A). However, vanadate with H_2O_2 exerted a much more potent inhibitory effects on GTPγS-induced [^{3}H]PEt formation. Vanadate (200 µM) with H_2O_2 (2 mM) inhibited GTPγS-induced [^{14}C]5-HT secretion by 32% but abolished [^{3}H]PEt accumulation stimulated by GTPγS. In the absence of ethanol, vanadate and H_2O_2, either individually or together, increased GTPγS-induced [^{3}H]DAG formation, though these effects were less than additive (Table 7B). Under the latter condition, vanadate (200 µM), either alone or with H_2O_2 (2 mM), again caused a significant inhibition of GTPγS-induced [^{14}C]5-HT secretion. These results do not correlate well with the effects of vanadate and H_2O_2 on either [^{3}H]PEt or [^{3}H]DAG formation.

3.5.6. Effects of a tyrosine kinase inhibitor on secretion and on [^3H]PEt and [^3H]DAG formation induced by vanadate/H_2O_2. To examine further the mechanism by which vanadate/H_2O_2 induces [^{14}C]5-HT secretion and the relationship with the activation of PLD and PLC, the effects of a specific tyrosine kinase inhibitor were examined in a preliminary experiment. ST271 was selected because this compound has been used to investigate the properties and functions of tyrosine kinases both *in vitro* and *in vivo*, including their role in the activation of PLD in human neutrophils (Shiraishi *et al.*, 1987; Uings *et al.*, 1992). At pCa 6, 100 µM ST271 partially inhibited [^{14}C]5-HT

secretion but much more markedly decreased the stimulation of PLD activity by vanadate (200 μM), H_2O_2 (2 mM) and vanadate (4 μM) with H_2O_2 (40 μM) (Table 8A). In the absence of ethanol, ST271 did not inhibit [^{3}H]DAG formation stimulated by H_2O_2 (2 mM) alone or vanadate (4 μM) with H_2O_2 (40 μM), but only decreased the [^{3}H]DAG accumulation caused by vanadate (200 μM) alone (Table 8B). Thus, only part of the secretion could be related to PLD activity. These preliminary results are, however, consistent with a role for a tyrosine kinase in the activation of PLD in platelets.

Table 7. Effects of vanadate/H_2O_2 on the secretion and formation of phospholipid metabolites induced by GTPγS 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 then incubated (10 min at 25°C) at pCa 6 with the indicated concentrations of vanadate, H_2O_2 and GTPγS in the presence (A) or absence (B) of ethanol (200 mM). Secretion of [^{14}C]5-HT and the formation of [^{3}H]PEt (A) or [^{3}H]DAG (B) were determined; values are means ± S.E. from triplicate samples.

A

Additions	Secretion of [^{14}C]5-HT (%)	[^{3}H]PEt formation (10^{-3}x d.p.m./10^{9} platelets)
No GTPγS		
None	8 ± 1	1.9 ± 0.4
H_2O_2 (2 mM)	67 ± 2	6.2 ± 0.3
Vanadate (40 μM)	64 ± 2	7.3 ± 0.1
Vanadate (200 μM)	64 ± 2	7.1 ± 0.4
Vanadate (40 μM) + H_2O_2 (2 mM)	66 ± 1	2.5 ± 0.1
Vanadate (200 uM) + H_2O_2 (2 mM)	54 ± 1	1.4 ± 0.4
With GTPγS (100 μM)		
None	79 ± 0	16.4 ± 0.9
H_2O_2 (2 mM)	76 ± 4	14.5 ± 0.8
Vanadate (40 μM)	76 ± 0	17.3 ± 0.5
Vanadate (200 μM)	70 ± 2	12.7 ± 0.5
Vanadate (40 μM) + H_2O_2 (2 mM)	69 ± 1	5.0 ± 0.1
Vanadate(200 μM) + H_2O_2 (2 mM)	54 ± 3	1.6 ± 0.1

B

Additions	Secretion of [^{14}C]5-HT (%)	[^{3}H]DAG formation (10^{-3}x d.p.m./10^{9}platelets)
No GTPγS		
None	20 ± 2	2.1 ± 0.1
H_2O_2 (2 mM)	71 ± 1	104.9 ± 2.4
Vanadate (40 μM)	68 ± 1	27.9 ± 5.8
Vanadate (200 μM)	67 ± 5	130.5 ± 1.6
Vanadate (40 μM) + H_2O_2 (2 mM)	71 ± 2	121.4 ± 3.5
Vanadate (200 uM) + H_2O_2 (2 mM)	55 ± 5	96.0 ± 1.2
With GTPγS (100 μM)		
None	77 ± 2	61.3 ± 2.1
H_2O_2 (2 mM)	79 ± 4	138.6 ± 1.2
Vanadate (40 μM)	76 ± 1	63.9 ± 1.7
Vanadate (200 μM)	55 ± 4	142.6 ± 3.4
Vanadate (40 μM) + H_2O_2 (2 mM)	72 ± 1	137.7 ± 5.7
Vanadate (200 μM) + H_2O_2 (2 mM)	60 ± 1	110.3 ± 3.4

Table 8. Effects of ST271 on vanadate/H_2O_2-induced secretion and on the associated formation of phospholipid metabolites 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 then incubated (10 min at 25° C) at pCa 6 with the indicated concentrations of vanadate and H_2O_2, with or without ST271 (100 µM) in the presence (A) or absence (B) of ethanol (200 mM). Secretion of [^{14}C]5-HT and the formation of [^{3}H]PEt (A) or [^{3}H]DAG (B) were determined; values are means ± S.E. from triplicate samples.

A

Additions	Secretion of [^{14}C]5-HT (%)		[^{3}H]PEt formation (10^{-3}x d.p.m./10^{9} platelets)	
	-ST271	+ST271	-ST271	+ST271
None	8 ± 1	4 ± 1	1.8 ± 0.0	- 0.3 ± 0.1
Vanadate (200 μM)	63 ± 1	58 ± 4	4.3 ± 0.2	0.6 ± 0.0
H_2O_2 (2 mM)	66 ± 1	45 ± 2	3.3 ± 0.2	1.1 ± 0.1
Vanadate (4 μM) + H_2O_2 (40 μM)	71 ± 1	57 ± 2	4.8 ± 0.1	1.0 ± 0.1

B

Additions	Secretion of [^{14}C]5-HT (%)		[^{3}H]DAG formation (10^{-3}x d.p.m./10^{9} platelets)	
	-ST271	+ST271	-ST271	+ST271
None	23 ± 2	8 ± 1	5.8 ± 0.9	5.9 ± 1.4
Vanadate (200 μM)	76 ± 1	58 ± 3	169.4 ± 3.2	108.7 ± 6.8
H_2O_2 (2 mM)	73 ± 3	54 ± 2	133.7 ± 0.6	131.2 ± 15.6
Vanadate (4 μM) + H_2O_2 (40 μM)	74 ± 1	57 ± 4	158.2 ± 4.6	171.0 ± 5.3

4. DISCUSSION

4.1. Relationships between secretion and PLD and PLC activation in platelet

Previous studies from this laboratory established the hypothesis that three factors (Ca^{2+}, PKC, GTP-binding proteins) are involved in the regulation of the secretion of both dense and α-granule constituents from electropermeabilized human platelets. A combination of any two of these three factors is required for marked secretion but none of the three factors is essential (reviewed by Haslam and Coorssen, 1993). Therefore identification of any common target of Ca^{2+}, PKC and GTPγS would provide evidence to support the above hypothesis. Studies have shown that both PLC and PLD are regulated by or contribute to the availability of these three factors and thus suggest that both PLC and PLD could play roles in exocytosis from platelets (Knight and Scrutton, 1984; Haslam and Davidson, 1984a,b,c; Culty *et al.*, 1988; Van der Meulen and Haslam, 1990; Coorssen *et al.*, 1990; Coorssen and Haslam, 1993). In this study, using KF/$AlCl_3$ and vanadate/H_2O_2 as stimuli, the relationships of the activation of PLC and PLD with exocytosis have been investigated in electropermeabilized human platelets. The results of this study provide evidence that activation of PLD or PLC can mediate marked secretion of dense granule contents from electropermeabilized human platelets. In these studies, [^{14}C]5-HT was used as a marker for dense granule secretion. Both PLD and PLC are involved in

the regulation of [^{14}C]5-HT secretion but have distinct contributions to Ca^{2+}-dependent and Ca^{2+}-independent [^{14}C]5-HT secretion. The study shows that the treatment of permeabilized platelets with KF/$AlCl_3$ results in a Ca^{2+}-dependent [^{14}C]5-HT secretion which correlates with the activation of PLC. The stimulatory effect of KF/$AlCl_3$ on PLC was demonstrated by [^{3}H]DAG formation and increased [^{3}H]inositol phosphate accumulation. KF/$AlCl_3$ also stimulated Ca^{2+}-dependent pleckstrin phosphorylation, indicating an activation of protein kinase C. These results support the view that fluoride or fluoroaluminate (AlF_4^-) induces a Ca^{2+}-dependent secretion through the activation of PLC and are consistent with the concept that activation of PLC and PKC plays an important role in Ca^{2+}-dependent secretion (Nishizuka, 1984; Haslam and Davidson 1984a). They are also in agreement with some previous studies on the effects of fluoride on intact platelets, which demonstrate the activation of PLC and PKC (Kienast *et al.*, 1987; Pfliegler *et al.*, 1993; Lazarowski *et al.*, 1989; Doni *et al.*, 1988).

Although KF/$AlCl_3$ did not stimulate PLD activity at either pCa > 9 or pCa 6, it inhibited GTPγS-stimulated PLD activity. Only in the absence of Ca^{2+}, did the inhibitory effects of KF/$AlCl_3$ on secretion induced by GTPγS alone or GTPγS plus PMA correlate well with its inhibitory effects on PLD activity. These results support the view that the target of G_E in permeabilized platelets is PLD (Gomperts, 1990) and are consistent with the previous evidence that PLD activity correlated well with Ca^{2+}-independent secretion (Coorssen and Haslam, 1993). In the presence of Ca^{2+} (pCa 6), the inhibitory effects of KF/$AlCl_3$ on secretion induced by GTPγS correlated

with its inhibitory effects on PLC rather than on PLD, suggesting that although both PLD and PLC are involved in Ca^{2+}-dependent secretion, PLC is likely to be more important. The experiments with vanadate/H_2O_2 also support the idea that PLC is more important for Ca^{2+}-dependent secretion than PLD. Thus, the results show that in the presence of Ca^{2+} (pCa 6), the effects of vanadate/H_2O_2 on [^{14}C]5-HT secretion correlated with [^{3}H]DAG formation but not for with [^{3}H]PEt formation. In addition, although GTPγS-induced PLD activity was abolished by vanadate/H_2O_2, GTPγS-induced secretion was only partially inhibited, implying that PLD could account for only part of the Ca^{2+}-dependent secretion.

The results from this study also indicated that the activation of PLD differs markedly from that of PLC in permeabilized platelets. First, KF/$AlCl_3$ could stimulate PLC activity, but failed to stimulate PLD. The inhibitory effect of KF/$AlCl_3$ on GTPγS-induced PLD activity was stronger than that on GTPγS-induced PLC activity. Second, in the absence of Ca^{2+}, PLD was synergistically activated by GTPγS and PMA, but there was no PLC activation; at pCa 6, PMA inhibited PLC activation but not that of PLD. Third, vanadate with H_2O_2 synergistically stimulated PLC activity but had a biphasic effect on PLD activity. Further, tyrosine kinase inhibitor ST271 markedly inhibited PLD activity but had little effects on PLC activity. Furthermore, the results suggest that KF/$AlCl_3$ induced a Ca^{2+} and PLC-dependent secretion, probably through the activation of a heterotrimeric G protein, although alternative mechanisms can not be easily excluded (e.g. inhibition of protein phosphatases). The results also suggest that the guanine nucleotide binding protein

that activates PLC is distinct from the protein that activates PLD in permeabilized platelets. It is likely that GTPγS activates platelet PLD via an unidentified low-M_r GTP-binding protein, since KF/$AlCl_3$ was inhibitory not stimulatory. This is consistent with the observations in HL60 cells where ARF has been identified as an activator of PLD (Brown *et al.*, 1993; Cockcroft *et al.*, 1994), and with that in human neutrophils where a rho family low-M_r GTP-binding protein was thought to stimulate PLD (Bowman *et al.*, 1993). Based on these observations and previous studies on electropermeabilized platelets, the regulation of exocytotic mechanism is summarized in Fig. 16.

4.2. *Effects of KF/$AlCl_3$ on secretion and on PLC and PLD activities*

4.2.1. Effects of KF/$AlCl_3$ on [^{14}C]5-HT secretion. In this study, it was shown that KF/$AlCl_3$ caused a Ca^{2+}-dependent 5-HT secretion. Maximal stimulation occurred with 10 mM KF and 10 μM $AlCl_3$ at pCa 6; higher concentrations (20 mM KF and 20 μM $AlCl_3$) had a similar effect. The concentrations of KF/$AlCl_3$ used in these studies were not intended to mimic any physiological condition but rather were used to provide a selective agonist for study of the secretory mechanism in permeabilized platelets. Mürer (1986) first observed that NaF (10 mM) could slowly induce the release of adenine nucleotides from intact human platelets. Later, maximal aggregation and secretion of ATP were observed in the presence of 30 mM KF (Kienast *et al.*, 1987). It was also shown that in intact human platelets, addition of

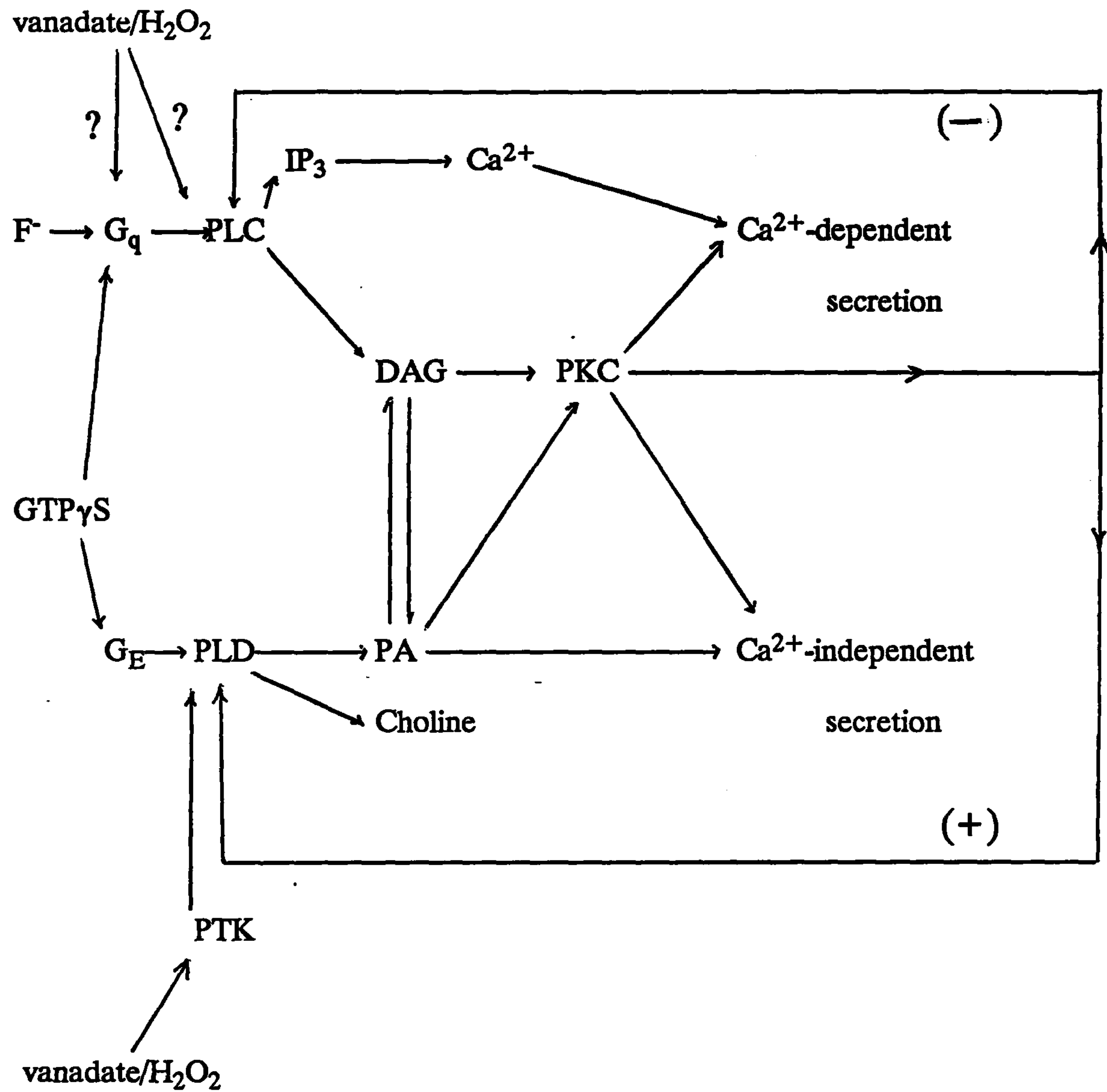


Fig. 16. Proposed mechanism for the regulation of exocytosis triggered by F^-, GTPγS and vanadate/H_2O_2 in electropermeabilized human platelets.

10 mM NaF and 10 μM $AlCl_3$ resulted in aggregation as well as 20% secretion of 5-HT (Rendu *et al.*, 1990). As observed in other cells or tissues, F^- at a concentration of 10-20 mM with or without micromolar concentrations of aluminum is sufficient to cause cellular activation. For example, maximal stimulation of IP formation in hamster fibroblasts was obtained with 10 mM NaF and 3 μM $AlCl_3$ (Paris and Pouyssegur, 1987). Also in digitonin-permeabilized human neuroblastoma cells, 10 mM NaF caused a maximal IP formation, whereas in the intact cells, a maximal activation was obtained with 15-20 mM NaF (Fisher *et al.*, 1993). Studies in intact human platelets have shown that there was no release of cytosolic lactate dehydrogenase when the concentrations of NaF were lower than 20 mM, indicating no lysis of the platelets occurred (Rendu, 1990). Experiments with intact human platelets as well as other cells, such as hepatocytes, neutrophils and mast cells, demonstrated that NaF achieved its full effect in only minutes (Pfliegler *et al.*, 1993). Thus, in the present study, 10 min incubations (at 25°C) were chosen. This incubation time has been proved to be sufficient for stimulation of secretion by Ca^{2+} or GTPγS and therefore has been used routinely (Haslam and Davidson, 1984a, b, c).

4.2.2. Mechanism of action of $KF/AlCl_3$. Based on the different equilibrium constants, the major complex of Al^{3+} and F^- is AlF_4^- in the range of 1 - 50 mM F^- (Goldstein, 1964). AlF_4^- was proposed to be a phosphate analogue, since fluoride has an oxygen-like Van der Waals radius of 1.35 Å and the length of Al-F bond is

1.65 - 1.70 Å, whereas the length of P-O bond in PO_4^{3-} is 1.55 - 1.60 Å (Bigay *et al.*, 1985). Also, the hydrogen bond formed by the oxygen of the phosphate group could be obtained with F^-. Early studies showed that micromolar concentrations of Al^{3+} were required for F^- activation of G_s, G_i and transducin. Mg^{2+} and GDP are required for the AlF_4^--mediated G protein activation (Sternweis and Gilman, 1982; Katada *et al.*, 1984; Bigay *et al.*, 1985). Studies with hamster fibroblasts demonstrated that 2 - 3 μM $AlCl_3$ could cause the same maximal potentiation of 10 mM F^--induced inositol phosphate formation as 10 μM $AlCl_3$ (Paris and Pouyssegur, 1987). In that experiment, the concentration of Mg^{2+} was 1 mM. In isolated hepatocytes, $AlCl_3$ (10 μM) potentiated the effects of low concentrations of NaF (2-15 mM) on Ca^{2+} mobilization, activation of phosphorylase and inhibition of cAMP accumulation (Blackmore *et al.*, 1985). The Al^{3+} chelator, deferoxamine, blocked these effects (Blackmore *et al.*, 1985). However, the concentration of Mg^{2+} used in these experiments is unclear and deferoxamine also inhibited the effect of NaF alone. In the present studies, addition of micromolar concentrations of $AlCl_3$ to F^- solutions at millimolar concentrations should be sufficient for the formation of the AlF_4^- complex. However, it was shown that addition of Al^{3+} caused no potentiation of KF-induced secretion (Table 2). One possible explanation is aluminum contamination derived from commercial reagents or laboratory glassware. KF itself at 10 mM contained about 4 μM aluminum and, in the glutamate buffer system, about 10 μM aluminum was contributed by the other compounds present, such as ATP, EGTA or EDTA. The maximum concentration of aluminum under my experimental conditions is close

to 38 μM if both added $AlCl_3$ (20 μM) and contaminating aluminum (about 18 μM) are included. This amount of aluminum is sufficient for the formation of AlF_4^- (Goldstein, 1964), and calculations using a computer program based on that of Fabiato and Fabiato (1979) show that this amount of aluminum does not significantly affect the pCa value of the buffer system used and only slightly affects the concentrations of Mg^{2+} and ATP. However, added Al^{3+} had no effect on [^{14}C]5-HT secretion caused by 1-20 mM F^-. Moreover, after addition of the aluminum chelator deferoxamine, the effects of KF were not changed (M.M.L. Davidson personal communications). One possible other explanation is that the effects of KF in this system are Al^{3+}-independent. Effects of F^- also have been observed by others in the absence of aluminum ions. Thus, in human neuroblastoma cells, NaF was found to stimulate inositol phosphate formation via the activation of a G protein without the addition of aluminum (Fisher *et al.*, 1991). The involvement of a G protein was demonstrated by using U-73122, a novel aminosteroid inhibitor of guanine nucleotide-regulated PLC activity or by comparison of the effects of NaF with those of GTPγS and GDPβS. It is now known that G proteins can be activated by F^- without Al^{3+} (Antonny *et al.*, 1990). The most likely explanation for actions of F^- in the absence of aluminum is the presence of Mg^{2+}. Recently, ^{31}P-NMR studies showed that Gα[GDP] binds 2 Mg^{2+} and 3 F^- ions to form the active compound Gα[GDP-MgF_3^-]Mg, which is structurally analogous to Gα[GDP-AlF_x]Mg and Gα[GTP]Mg (Antonny *et al.*, 1993). In the activation of muscarinic atrial K^+ channels, it was found that Al^{3+} enhanced the effects of low concentrations of KF (1 mM) in the presence of

low concentrations of Mg^{2+} (2 mM); however, no significant potentiation by Al^{3+} was observed with high concentrations of KF (10 mM) in the presence of 2 mM or 20 mM Mg^{2+} (Yatani and Brown, 1991). The concentration of free Mg^{2+} in the experimental system used in this thesis was 5 mM which might be sufficient for the formation of the $G\alpha[GDP\text{-}MgF_3]Mg$ complex in the absence of Al^{3+}. In previous studies using F^- and Al^{3+}, the concentrations of Mg^{2+} have been quite variable, ranging from 0.5 mM to more than 5 mM (Rendu at al., 1990; Kawase and Breemen, 1992). It is at present controversial whether a requirement of Al^{3+} for an effect of F^- reflects the involvement of a G-protein, since F^- could activate a G-protein without Al^{3+} and F^- (or AlF_4^-) may have multiple sites of action in mammalian tissues.

4.2.3. Comparison the effects of KF/AlCl$_3$ with GTPγS. In this study, although there is no direct evidence that the mechanism of action of KF/$AlCl_3$ is via the activation of G-proteins, comparison of the effects of KF/$AlCl_3$ on secretion, as well as on phospholipase activities (PLD and PLC), with those of GTPγS and PMA, may provide indirect evidence, since both PLD and PLC have been found to have roles in the pathways leading to secretion and both enzymes are regulated by GTPγS and PMA.

In the absence of Ca^{2+}, the effects of KF/$AlCl_3$ on [^{14}C]5-HT secretion with PMA were similar to but much weaker than those of GTPγS with PMA (Figs. 2 and 3A). Also KF/$AlCl_3$ inhibited the effects of high GTPγS concentrations on [^{14}C]5-HT secretion (Table 3, Figs. 4 and 5). In the presence of Ca^{2+}, the inhibitory effects of

$KF/AlCl_3$ on GTPγS-induced [^{14}C]5-HT secretion correlated well with its inhibitory effects on GTPγS-induced [^{3}H]DAG formation (Fig. 12). Alone, $KF/AlCl_3$ had stimulatory effects on secretion as well as on PLC that were much weaker than those of GTPγS (Fig. 11). These results suggested that $KF/AlCl_3$ might interact competitively with the same GTP-binding protein as GTPγS. In effect, $KF/AlCl_3$ appears to behave as a partial agonist for PLC activation and secretion. However, if the same G protein is involved in the activation of PLC by both GTPγS and $KF/AlCl_3$, these two stimuli have distinct effects. The low efficacy of $KF/AlCl_3$ on secretion and PLC activation as compared to GTPγS might also reflect some essential difference in the two activation pathways. The possibility also remains that there might be only a small fraction of the G proteins with a conformation accessible for $KF/AlCl_3$ binding. Alternatively, based on the hypothesis that $KF/AlCl_3$ functions as AlF_x (3-5) which binds to Gα[GDP] to form Gα[GDP-AlF_x^-]Mg^{2+}, there may be an equilibrium between these two forms. The effects of AlF_4^- on G protein activation are rapidly reversible and can be lost easily. It was found that AlF_4^- blocked GDP dissociation from Gα (Kahn, 1991) and this dissociation of GDP was required for activation of G_α by GTPγS or GTP but not for activation of G_α by AlF_4^- (Ferguson *et al.*, 1986). This may be an explanation for the inhibitory effects of $KF/AlCl_3$ on GTPγS via binding to the same G protein.

4.2.4. Effect of $KF/AlCl_3$ on PKC, PLC activation and its relationship with Ca^{2+}-dependent [^{14}C]5-HT secretion. Ca^{2+} is required for the [^{14}C]5-HT secretion from

permeabilized platelets induced by $KF/AlCl_3$. However, on increasing the Ca^{2+} concentration to pCa 5, $KF/AlCl_3$ has no additional effects on the secretion (about 80%, result not shown) because Ca^{2+} alone induces a near maximal secretion of both dense and α-granule constituents. Similarly, activation of GTP-binding proteins by GTPγS or of PKC by PMA shifted the Ca^{2+} concentration-response curve to the left and had little effect on the maximum secretion (Knight *et al.*, 1984; Haslam and Davidson, 1984c; Coorssen *et al.*, 1990). Therefore, it is possible that activation of a G protein or PKC or both could account for this Ca^{2+}-dependent [^{14}C]5-HT secretion promoted by $KF/AlCl_3$. Indications of a role for PKC in secretion have been obtained by measuring the phosphorylation of pleckstrin (P47) or the effects of PMA, a well known PKC activator. $KF/AlCl_3$ also caused a Ca^{2+}-dependent phosphorylation of pleckstrin and the maximal effect was obtained under the same conditions as maximal secretion (Fig. 1.). This result suggests that the activation of PKC may be involved in the $KF/AlCl_3$-induced secretion. In the absence of Ca^{2+} (pCa > 9), neither PKC activation nor secretion occurred in response to $KF/AlCl_3$. In the presence of Ca^{2+} (pCa 7 and 6), the effects of $KF/AlCl_3$ and PMA on secretion were less than additive (Fig. 3A), suggesting a common site of action. These results have confirmed the concept that the presence of both Ca^{2+} and of PKC activation can mediate an optimal secretion from permeabilized platelets (Haslam and Coorssen, 1993). However, the activation of PKC cannot be the final common pathway leading to secretion. As shown in Fig 1., the pleckstrin phosphorylation caused by 10 mM KF/ 10 μM $AlCl_3$ at pCa 7 was similar to the

corresponding effect of 4 mM KF/4 μM $AlCl_3$ at pCa 6, though their effects on [^{14}C]5-HT secretion were different. In addition, the effects of KF/$AlCl_3$ on GTPγS- or PMA- stimulated secretion did not correlate well with its effects on GTPγS- or PMA- stimulated pleckstrin phosphorylation in the presence of Ca^{2+} (Table 3). Thus, the secretion was not solely PKC-dependent. The mechanism by which KF/$AlCl_3$ induced [^{14}C]5-HT secretion from permeabilized platelets cannot be attributed to a direct interaction between KF/$AlCl_3$ and PKC. There is no evidence showing that KF/$AlCl_3$ can act directly on PKC, but it is possible that the effect of PKC might be enhanced by inhibition of protein phosphatases by F^-. However, okadaic acid, which inhibits protein phosphatases 1 and 2A, enhances rather than inhibits GTPγS-induced secretion at pCa > 9 (Davidson and Haslam, 1994). Although early studies from this laboratory established that Ca^{2+} and the guanine nucleotide-dependent activation of PLC and PKC are essential for secretion from permeabilized platelets (Haslam and Davidson, 1984a,b,c), studies in mast cells (Gomperts, 1990) have indicated that Ca^{2+} and guanine nucleotide rather than PKC directly regulate exocytosis. In addition, studies from this laboratory using PKC inhibitors have shown that GTPγS could induce secretion in the absence of PKC activity, provided a high Ca^{2+} concentration (pCa 4.5) was present (Haslam and Coorssen, 1993). Since F^- has been shown to activate G proteins in the presence of Al^{3+}, the effect of KF/$AlCl_3$ on secretion is most likely to be explained by a pathway involving the activation of G proteins other than G_E.

There is evidence that low concentrations of NaF (< 10 mM) inhibit the Ca^{2+}

influx into intact platelets induced by receptor-mediated platelet activation, such as that caused by thrombin or a thromboxane A_2 analogue (Ozaki *et al.*, 1993). It has also been found that there was no increase in cytoplasmic [$Ca^{2+}{}_{free}$] after incubation of intact human platelets with 10 mM NaF and subsequent addition of $AlCl_3$ (Rendu *et al.*, 1990). The present study would not detect an action of KF on Ca^{2+} influx, since the platelets were permeabilized.

Previous studies demonstrated that NaF had a Li^+-like effect on inositol phosphate formation induced by thrombin in hamster fibroblasts (Paris and Pouyssegur, 1987). F^- inhibited IP phosphatase and this effect was not dependent on Al^{3+}. However, Li^+ is a more potent inhibitor of IP phosphatases than is F^-, and did not cause any significant IP accumulation in permeabilized platelets. It is unlikely that the effects of KF/$AlCl_3$ on IP and IP_2 formation in permeabilized platelets can be fully accounted for by inhibition of these phosphatases, because there was no enhancement of the effect of GTPγS. In fact, KF/$AlCl_3$ inhibited GTPγS-induced IP formation but not that of IP_2. The effects of KF/$AlCl_3$ on inositol phosphate formation (in the absence and presence of GTPγS) were similar to its effects on DAG formation, suggesting that the main action of KF/$AlCl_3$ was on PLC rather than on inositol phosphatases. PMA abolished basal DAG formation and inhibited KF/$AlCl_3$-induced DAG formation, suggesting that protein kinase C exerts a negative feedback effect, either through the G protein or PLC itself. Inositol phosphate formation has been observed in intact platelets upon activation by NaF (Kienast *et al.*, 1987; Lazarowski *et al.*, 1989; Pfliegler *et al.*, 1993). Collectively, these results

suggest that G protein activation by KF/$AlCl_3$ mediates the activation of PLC.

4.2.5. Effect of KF/$AlCl_3$ on PLD activity and its relationship to Ca^{2+}-independent [^{14}C]5-HT secretion. In this study, in contrast to its stimulation on PLC, KF/$AlCl_3$ failed to stimulate PLD. Although it is possible that the assay used to detect [^{3}H]DAG formation is much more sensitive than that for [^{3}H]PEt formation, comparison the effects of KF/$AlCl_3$ with low concentrations of GTPγS (1-2 μM) provided evidence that there is a significant difference in their effects on [^{3}H]PEt formation. Although KF/$AlCl_3$ did not stimulate either secretion or PLD activity in the absence of Ca^{2+}, its inhibitory effects on secretion induced by GTPγS alone or GTPγS plus PMA correlated well with its inhibitory effect on PLD activity (Fig. 6). This result is in agreement with the previous studies showing that PLD activation was associated with [^{14}C]5-HT secretion (Coorssen and Haslam, 1993), and supports the view that PLD plays an important role in Ca^{2+}-independent secretion. In the absence of Ca^{2+}, there was no detectable [^{3}H]DAG formation in response to KF/$AlCl_3$ (Fig. 10B), suggesting that PLC has no significant role in Ca^{2+}-independent secretion. This is also in agreement with previous observations that there is no inositol phosphate or DAG formation in GTPγS-treated permeabilized platelets at pCa>9 (Coorssen *et al.*, 1990; Haslam and Coorssen, 1993). A contribution of PLA_2 to this Ca^{2+}-independent secretion can also be ruled out because at pCa>9, inhibition of PLA_2 did not affect secretion from permeabilized platelets (Coorssen, Ph.D. thesis, 1993). These observations further support a pivotal role of PLD in the

regulation of Ca^{2+}-independent secretion. The mechanism of $KF/AlCl_3$ inhibition of PLD is uncertain; it is possible that $KF/AlCl_3$ may inhibit PLD activity by inhibiting the binding of GTPγS to the PLD regulatory GTP-binding protein. Although inhibitory effects on PA formation in previous studies with hepatocyte membranes suggested that F^- may have a direct inhibitory effect on PLD (Bocckino *et al.*, 1987), this has not been demonstrated with purified PLD. In permeabilized platelets, $KF/AlCl_3$ had little effect on basal PLD activity or on PMA-induced PA formation, also implying that a direct action of F^- on PLD is unlikely. Furthermore, based on the fact that GTPγS can stimulate both low-M_r GTP-binding proteins and heterotrimeric G proteins (Oberhauser *et al.*, 1992), our results suggest that PLD activation in permeabilized platelets could be mediated by a low-M_r GTP-binding protein, rather than by a heterotrimeric G protein. Thus, the unidentified G_E might be belong to the family of low-M_r GTP-binding proteins. This view is consistent with results showing that AlF_4^- inhibits GTP-dependent vesicle fusion in rat liver microsomes and blocks GTPγS binding to G_n proteins (Comerford and Dawson, 1991), defined as the low-M_r GTP-binding proteins that bind [α-^{32}P]GTP on nitrocellulose blots (Bhullar and Haslam, 1987). If G_E is a low-M_r GTP-binding protein, the inhibitory effects of AlF_4^- or F^- on GTPγS-induced PLD activity is consistent with a role for PLD in secretion. Alternatively, based on the fact that AlF_4^- and GTPγS both inhibit intracellular traffic between the endoplasmic reticulum and Golgi and within the Golgi complex, it has been suggested that a heterotrimeric G protein may be involved in the regulation of vesicular transport (Pfeffer, 1992).

It has also been suggested that G proteins might be involved in fusion of intracellular organelles, rather than in exocytosis at the plasma membrane (Knight *et al.*, 1989). Since heterotrimeric G proteins are also located on the membranes of secretory granules, it is possible that the activation by AlF_4^- of one of these G proteins inhibits the exocytotic mechanism. Thus, the inhibitory effects of AlF_4^- on GTPγS-induced PLD activity might be due to an action of $KF/AlCl_3$ on a heterotrimeric G protein distinct from the GTP-binding protein involved in the activation of PLD.

It has been established that the ARF family of low-M_r GTP-binding proteins plays an important role in vesicular traffic. This view is based on the observations that ARF is essential for yeast protein transport (Stearns *et al.*, 1990), that ARF locates at Golgi-directed coated vesicles (Serafini *et al.*, 1991; Rothman and Orci, 1992), and that activated ARF (GTPγS-bound) inhibits fusion of Golgi, ER-Golgi, endosome-endosome and nuclear vesicles (Kahn *et al.*, 1994). ARF may exert its effects on vesicular traffic through the activation of PLD (Kahn *et al.*, 1993). PA accumulation on the donor membranes through the activation of PLD by ARF may cause the membranes to bud; this stimulated membrane may then become part of the vesicular traffic initiated or regulated by PA (Liscovitch and Cantley, 1994). A role for PLD and PA in the regulation of fusion or fission processes in vesicular trafficking has also been proposed because ethanol inhibits secretion in many cell types, such as HL-60 cells, neutrophils and platelets (Stutchfield and Cockcroft, 1993; Haslam and Coorssen, 1993). The inhibitory effect of ethanol is due to the decrease of PA production by PLD through the competitive transphosphatidylation reaction at the

expense of PA (Yang *et al.*, 1967). Thus, secretion appear to be inhibited by diversion of PA to PEt. A similar mechanism may account for the inhibitory effect of ethanol on coated vesicles and buds in vesicular transport (Pfanner *et al.*, 1989).

4.3. Effects of vanadate/H_2O_2 on secretion and on PLC, PLD activities

In permeabilized platelets, vanadate or H_2O_2 stimulates Ca^{2+}-dependent [^{14}C]5-HT secretion and PLC activation at lower concentrations than F^-. The effects of vanadate and/or H_2O_2 on PLD activation and platelet protein phosphorylation are quite different from those of KF/$AlCl_3$. In the presence of Ca^{2+}, either vanadate or H_2O_2 is a potent stimulator of both PLC and PLD activities which correlated with the stimulation of [^{14}C]5-HT secretion. However, when used together, the synergistic effects of vanadate and H_2O_2 on [^{14}C]5-HT secretion only correlated with its effects on PLC activation, but not on PLD activation, supporting the idea that PLD is not essential for Ca^{2+}-dependent secretion. Vanadate caused an almost maximal secretion (72%) of [^{14}C]5-HT at 40 µM. In the presence of H_2O_2, much lower concentrations of vanadate caused maximal [^{14}C]5-HT secretion as well as PLC activation. Even 0.4 µM vanadate and 4 µM H_2O_2 caused 73% secretion of [^{14}C]5-HT (data not shown). Since low concentrations of vanadate (0.1 - 1.0 µM) are found naturally in the sera and tissues of vertebrates (Cantley *et al.*, 1979), this observation raises the possibility that vanadate is a physiological modulator of PLC and PLD. Vanadate was shown to stimulate the microsomal oxidation of NAD(P)H, which generates active oxygen species (O_2^-) leading H_2O_2 formation. H_2O_2 is widely

generated in cellular plasma membranes and has been proposed to be a purposeful product instead of a mere byproduct of cell respiration (reviewed by Ramasarma, 1982). Exogenous H_2O_2 (200 nM) has been shown to enhance platelet aggregation stimulated by agonists, and during platelet activation, generation of H_2O_2 was induced inside platelets by the same agonists (Principe *et al.*, 1985). However, the mechanism responsible for the effects of H_2O_2 was not clear, although H_2O_2 could be an intermediate in a cascade process and endogenous vanadate might be required. Various oxidation states of vanadium exist in cells. It was found that in human red cells, vanadate in the +5 oxidation state is much more effective than vanadate in the +4 oxidation state as an inhibitor of (Na^+-K^+)ATPase (Cantley and Aisen, 1979). Synergism of vanadate and H_2O_2 has also been observed in stimulation of IGF-II binding to rat adipocytes and of insulin receptor kinase (Kadota *et al.*, 1987). It has been demonstrated that in the presence of H_2O_2, orthovanadate forms pervanadate which can account for the synergistic insulin-like effect of vanadate and H_2O_2. Pervanadate is 10^2-10^3 times more potent than vanadate as an insulin mimetic and has inhibitory effects on phosphotyrosine phosphatase with a specificity distinct from that of vanadate (Fantus *et al.*, 1989).

The mechanism by which vanadate/H_2O_2 stimulates PLC is far from clear. The stimulatory effect of vanadate on PLC could be mediated by vanadate or pervanadate through interactions with: (1) receptors which couple to G_q or receptors which have intrinsic protein tyrosine kinase activity and couple to PLCγ; (2) G_q; (3) PLC itself; (4) tyrosine phosphatases. Although it is hard to exclude the possibility

of an action of vanadate involving a receptor, it is possible that vanadate can easily enter into permeabilized cells by bypassing the plasma membrane, and that H_2O_2 could facilitate its entry (Inazu *et al.*, 1990). In other cells stimulated by vanadate/H_2O_2, it was found that the formation of inositol phosphates correlated well with protein tyrosine phosphorylation (Zick and Sagi-Eisenberg, 1990). Tyrosine phosphorylation of proteins with different molecular masses had different correlations with IP or IP_3 formation (Zick and Sagi-Eisenberg, 1990). However, in permeabilized platelets, the receptors leading to PLCγ activation or protein tyrosine phosphorylation may not be involved, since a non-specific tyrosine kinase inhibitor (ST271) did not inhibit vanadate/H_2O_2-stimulated PLC activity. Studies in hamster fibroblasts showed that vanadate stimulated PLC activation and that this effect of vanadate was inhibited by pertussis toxin (Paris and Pouyssegur, 1987). Thus, it was suggested that vanadate activated PLC by direct activation of the regulatory G protein. Also Krawietz (1982) provided evidence for the idea that vanadate activation of adenylyl cyclase involves G proteins, since vanadate was shown to inhibit basal- and isoproterenol-stimulated GTPase activity in turkey erythrocyte membranes. Maximal stimulation by vanadate in turkey erythrocyte membrane was obtained at 3 mM. Alternatively, vanadate might exert an inhibitory effect on a phosphatase which would not by itself cause a significant activation but could synergistically amplify the effects of a protein kinase that promotes activation of phosphoinositide hydrolysis.

A biphasic effect of increasing concentrations of vanadate with H_2O_2 on PLD

activity has not been observed previously. Vanadate/H_2O_2 at very low concentrations synergistically stimulated PLD activity but then inhibited PLD activity at higher concentrations. The basis for the stimulation and inhibition of PLD by vanadate/H_2O_2 is unknown. These effects may involve interactions with components on the pathway leading to the activation of PLD, interactions with the PC binding site on PLD or some less specific effect. Further studies are necessary to define the mechanism of inhibition of PLD by high concentrations of vanadate/H_2O_2. However, the inhibition of PLD activity by high vanadate/H_2O_2 concentrations, could be due to the formation of reactive oxygen species, as the result of activation of PKC, protein tyrosine kinase and NADPH oxidase (Zor *et al.*, 1993). Vanadate/H_2O_2 may increase PLD activity through amplification of the effects of a protein tyrosine kinase, since vanadate/H_2O_2 is a potent inhibitor on protein tyrosine phosphatase. In this as in previous studies (Inazu *et al.*, 1990), vanadate/H_2O_2 demonstrated the ability of enhance the phosphorylation of several proteins including two of 53 and 38 kDa, which have been identified by phosphotyrosine immunoblotting assays as protein-tyrosine kinase substrates in intact human platelets (Inazu *et al.*, 1990). It is therefore possible that protein-tyrosine phosphorylation is associated with PLD activation, as found in human neutrophils (Uings *et al.*, 1992). This conclusion is supported by the finding that ST271 inhibited [^{3}H]PEt formation. ST271 is a specific tyrosine kinase inhibitor and has little effect on serine and threonine protein kinases, such as PKA and PKC (Shiraishi *et al.*, 1989). Vanadate/H_2O_2 also markedly stimulated P47 phosphorylation, suggesting it stimulated PKC. Presumably, this was secondary to the

activation of PLC. Previous studies have shown that vanadate/H_2O_2 stimulated the activation and translocation to the plasma membrane of PKC and PLA_2 (Goldman *et al.*, 1992).

4.4. In closing

In summary, this study shows that both KF/$AlCl_3$ and vanadate/H_2O_2 can induce a Ca^{2+}-dependent [^{14}C]5-HT secretion which correlates with the activation of PLC in electropermeabilized human platelets. KF/$AlCl_3$ failed to stimulate PLD activation either in the presence or in the absence of Ca^{2+}. The inhibitory effects of KF/$AlCl_3$ on GTPγS- or GTPγS and PMA- stimulated PLD activity correlated with their effects on [^{14}C]5-HT secretion in the absence of Ca^{2+}, confirming the concept that PLD activity is involved in the regulation of Ca^{2+}-independent secretion. Since KF/$AlCl_3$ was inhibitory not stimulatory, these results also suggest that an unidentified low-M_r GTP-binding protein may be involved in the GTPγS-induced PLD activation in permeabilized platelets. Additional studies are necessary to identify this putative low-M_r GTP-binding protein. The simplest approach to this question would involve the use of functional domain peptides or of antibodies to specific low-M_r GTP-binding proteins (e.g. ARF) in streptolysin O-permeabilized platelets. Electropermeabilized platelets which only admit small molecules could not be used in the latter studies. An interesting finding in the present work is that vanadate in the presence of H_2O_2 had synergistic effects on stimulation of [^{14}C]5-HT secretion and PLC activation, but had a biphasic effect on PLD activation. Further

studies on this could involve the use of additional tyrosine kinase inhibitors to confirm the proposed role for a tyrosine kinase in the activation of PLD and the relative lack of an inhibitory effect on PLC activation. If reactive oxygen species are involved in the biphasic effects of vanadate and H_2O_2 on PLD activity, addition of superoxide dismutase or diphenyleneiodonium at the same time as vanadate and H_2O_2 could abolish their effects. To identify that PLD is the direct target of vanadate/H_2O_2, studies could be carried out with isolated platelet membranes. However, the mechanisms by which vanadate/H_2O_2 regulates PLD or PLC activity and the potential utility of this combination as a physiological or pharmacological agent remain to be determined.

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