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Regulation of platelet phospholipase C

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We have investigated factors affecting the activation of phospholipase C in human platelets. Prior exposure of platelets to phorbol esters that stimulate protein kinase C inhibits the activation of phospholipase C in response to a variety of receptor-directed agonists, including α- and γ-thrombin and thromboxane A_2 analogues. Such activation has been assayed by measurements of accumulated InsP_3 (including Ins(1,4,5)P_3 and Ins(1,3,4)P_3) and PtdOH. Inhibition is not overcome by Ca^{2+} ionophores, and substances that block or mimic Na^+–H^+ exchange neither block nor mimic these inhibitory effects. Cyclic AMP and cyclic GMP, other agents known to inhibit phospholipase C activation, do not accumulate in platelets exposed to phorbol esters. Although a portion of the effects of phorbol ester on InsP_3 accumulation may be explained by 5-phosphomonoesterase activity, it is likely that more direct effects on phospholipase C are being exerted as well, and contribute the major inhibitory route.

We have examined the susceptibility of adenylyl cyclase-associated G_i and 'G_p'-activated phospholipase C to inhibitory ADP-ribosylation by pertussis toxin-derived enzyme (S_1 protomer) administered to saponin-permeabilized platelets. The effects of α-thrombin on adenylyl cyclase can be inhibited by up to 50% by S_1, at which point inhibition of phospholipase C is barely detectable. Thromboxane A_2 analogues, which do not affect adenylyl cyclase (G_i), stimulate phospholipase C; this effect is not impaired by S_1. We therefore propose that the inhibitory effects of phorbol esters on the activation of phospholipase C are not mediated primarily by effects on G_i.

Introduction

Platelets are membrane-bound, anucleate bodies that normally circulate in discoid form in the blood stream. Their major function *in vivo* is to restrict the flow of blood from vascular lesions. They accomplish this by adhering to discontinuities in blood vessels, protruding pseudopods, recruiting and adhering to other platelets, secreting vasoconstrictive substances, providing a surface for the conversion of procoagulant to coagulant protein, and contracting to form a compact clot (figure 1). One can induce many of these changes in platelets *in vitro* by causing a rise in cytoplasmic Ca^{2+} levels after the addition of Ca^{2+} ionophores. Widespread interest in the connection between phosphoinositide turnover and platelet activation has led investigators to study the effects on phosphoinositide metabolism of a variety of agonists and inhibitors. Indeed, numerous platelet agonists have been reported to cause rises in cytoplasmic Ca^{2+} levels and inositol phosphate generation. In keeping with other cells, permeabilized platelets or platelet membranes exposed to myoinositol-1,4,5 trisphosphate (Ins(1,4,5)P_3) can release Ca^{2+} from sequestered stores (Brass & Joseph 1985; O'Rourke *et al.* 1985). Ins(1,4,5)P_3 thus

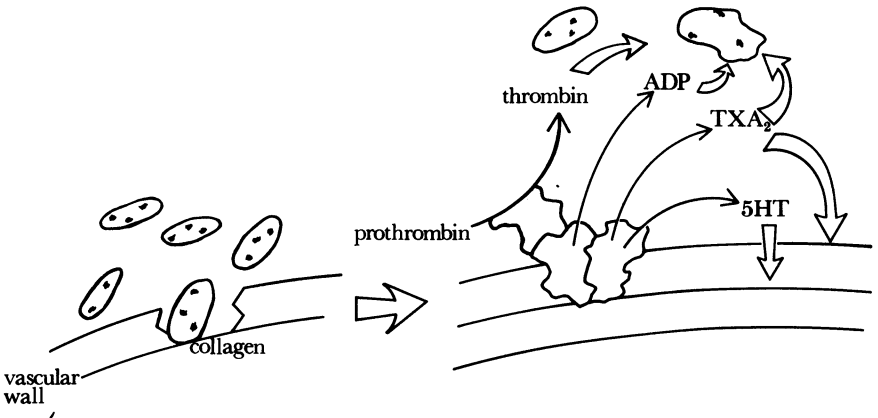

FIGURE 1. Activation of human platelets in the vascular system. Open arrows indicate agonist targets.

qualifies as a potentially important mediator of Ca^{2+} mobilization and of the ensuing cascade of Ca^{2+}-dependent activating responses in human platelets. Our intention in the course of this presentation is to dissect out some of the factors that regulate the generation of Ins(1,4,5)P_3 and therefore exert their effects at a critical early stage of platelet function. We shall focus particularly on the role of protein kinase C in this context.

AGONIST-INDUCED GENERATION OF InsP_3 IN PLATELETS

One of the most potent of platelet agonists is α-thrombin, a serine protease, displaying somewhat complex binding characteristics, which causes a rapid generation of InsP_3. Such InsP_3 has been assayed radioisotopically (Agranoff *et al.* 1983; Watson *et al.* 1984) and by mass (Rittenhouse & Sasson 1985). The majority of the mass of InsP_3 formed in response to

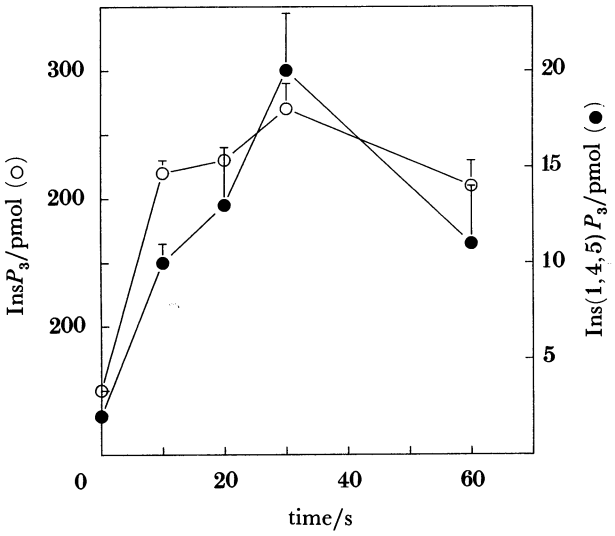

FIGURE 2. Different accumulations of InsP_3 and Ins(1,4,5)P_3 in platelets exposed to thrombin. All studies in this and succeeding figures were performed with aspirin-treated human platelets incubated at 37 °C. Platelets were exposed to α-thrombin (5 U ml^{-1}) for varied periods and the mass of InsP_3 or Ins(1,4,5)P_3 determined per 10^9 platelets (Tarver *et al.* 1987). A similar proportionality was observed for ^{32}P-labelled, thrombin-stimulated cells whose InsP_3 was resolved into Ins(1,3,4)P_3 and Ins(1,4,5)P_3 on a Partisil SAX (Whatman) column.

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thrombin is not Ins(1,4,5)P_3, but Ins(1,3,4)P_3, judging by its co-migration with Ins(1,3,4)P_3 standard during high-pressure liquid chromatography (HPLC) and failure to be acted upon by InsP_3 3-kinase (Tarver *et al.* 1987). Basal levels of Ins(1,4,5)P_3 in platelets are less than 0.2 μM, and rise within 10 s of the addition of thrombin to 1 μM (based on a platelet volume of 10 μl per 10^9 platelets), the bulk of which is the non-cyclic isomer. In contrast, Ins(1,3,4)P_3 levels rise from 2 μM to 22 μM in the same period (figure 2), an amount greater than that reported previously owing to improved techniques of recovery from platelet aggregates. Other platelet agonists, such as the thromboxane A_2 analogue ONO11113, also cause a skewed accumulation of Ins(1,3,4)P_3 relative to Ins(1,4,5)P_3. The early agonist-induced increases in Ins(1,4,5)P_3 are consistent with the concentrations necessary for Ca^{2+} mobilization, but, given the magnitude of the accumulation of Ins(1,3,4)P_3 noted above, it too may participate in Ca^{2+} mobilization (Irvine *et al.* 1986).

Activation of platelet protein kinase C

Platelet-stimulatory effects

With the discovery by Kishimoto *et al.* (1980) of Ca^{2+} and phosphatidylserine-dependent protein kinase C and its activation by either diacylglycerol (a byproduct, with Ins(1,4,5)P_3, of phospholipase C hydrolytic activity (Rittenhouse-Simmons 1979)) or tumour-promoting phorbol diesters (Castagna *et al.* 1982), attention focused initially on the possible role of protein kinase C in promoting platelet activation. It had been observed that phorbol myristate acetate (PMA) induced the fusion of membranes and secretion (Gerrard *et al.* 1977) and, later, that these effects and those of diacylglycerol were potentiated by Ca^{2+} (Kaibuchi *et al.* 1983). The mechanism by which this occurs has yet to be defined, but evidence that activation of C kinase is relevant to stimulation of platelets by physiologic agonists has been provided by Hannun *et al.* (1987). These investigators have shown that an inhibitor of C kinase, sphingosine, can inhibit the secretion and aggregation that is initiated ordinarily by collagen, γ-thrombin, ADP, or thromboxane A_2 derived from arachidonic acid. The stimulation of C kinase has been demonstrated as well to promote cytoplasmic alkalinization (Moolenaar *et al.* 1984; Siffert *et al.* 1987), and PMA has been reported to enhance the mobilization of arachidonic acid induced by Ca^{2+} ionophore in rabbit platelets (Touqui *et al.* 1986). It has been proposed that this implied enhancement of phospholipase A_2 action occurs by the inhibitory phosphorylation of lipocortin (considered to be a tonic endogenous inhibitor of phospholipase A (Touqui *et al.* 1986)). In contrast, Pollock *et al.* (1986), using comparable concentrations of PMA and ionomycin, have been unable to confirm this effect in human platelets.

Platelet-inhibitory effects

In 1985, however, several papers describing inhibitory effects on platelets of protein kinase C activation were published. MacIntyre *et al.* (1985) reported that prior incubation of platelets with 100 nM PMA or phorbol dibutyrate (PDBu) inhibited the rise in cytoplasmic Ca^{2+} and accumulation of phosphatidic acid (PtdOH, an indirect measure of diacylglycerol formation) which otherwise accompany the exposure of platelets to α-thrombin, vasopressin, or platelet-activating factor. Further, Zavoico *et al.* (1985) observed that the thrombin-induced transient decrease in PtdIns(4,5)P_2 was blocked by 100 nM PMA. De Chaffoy de Courcelles *et al.* (1984), however, had demonstrated a stimulation by PMA of phosphorylation of PtdIns and PtdIns4P,

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and Ca^{2+} efflux has been shown to be stimulated by PMA (Pollock *et al.* 1987). Because PMA, as noted above, may promote phospholipase A_2 action (a route for removal of PtdOH, albeit less likely in human platelets) and reduction of cytoplasmic Ca^{2+} levels can increase the utilization of PtdOH by cytidyl transferase, it was conceivable that the effects of phorbol ester were not due to inhibition at the level of phospholipase C. Additional findings make it likely that the activation of phospholipase C is affected as well. The levels of [^{3}H]inositol phosphates formed in response to thrombin, platelet-activating factor, or collagen are all depressed by pretreatment of platelets with 200 nM phorbol dibutyrate (PDBu (Watson & Lapetina 1985)). These inhibitory effects are also observed with respect to $InsP_3$ mass (Rittenhouse & Sasson 1985). As seen in figure 3, incubation of human platelets with PMA inhibits the accumulation

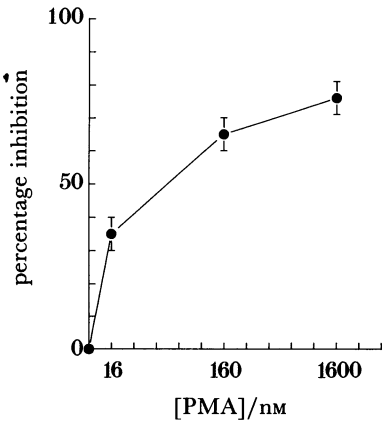

FIGURE 3. Inhibition by PMA of thrombin-induced $InsP_3$ formation in human platelets. Platelets were exposed to different concentrations of PMA for 5 min before the addition of 1 U ml^{-1} α-thrombin for 15 s, and $InsP_3$ mass was quantitated in comparison with inhibitor-free controls (Rittenhouse & Sasson 1985).

of $InsP_3$ in response to α-thrombin in a dose-dependent manner. γ-Thrombin, a product of the partial tryptic digestion of α-thrombin that lacks fibrinolytic activity and some other characteristics of α-thrombin, is none the less an activator of phospholipase C (measured as PtdOH (McGowan & Detwiler 1986) and measured as $InsP_3$ (table 1)). Its effects, too, are inhibited by phorbol diester (table 1), as are those of U46619, a prostaglandin H_2–thromboxane A_2 receptor-directed agonist (figure 4) and ONO11113, another thromboxane analogue (figure 5). Approximately 60% of the maximum inhibitory effect is observed within 15 s of the addition of 160 nM PMA and inhibition cannot be overcome by

TABLE 1. INHIBITION BY PMA OF $InsP_3$ ACCUMULATION IN HUMAN PLATELETS EXPOSED TO THROMBINS

(Aspirin-treated platelets were incubated with or without phorbol myristate acetate (PMA, 160 nM) for 5 min, followed by exposure to α-thrombin (2.5 nM, 0.2 U ml^{-1}) for 15 s or γ-thrombin (70 nM) for 45 s. The mass of $InsP_3$ was determined and values for agonist-free controls were subtracted.)

	Δ $InsP_3$/pmol		percentage inhibition with PMA
thrombin (type)	thrombin − PMA	thrombin + PMA	
α	84	14	88
γ	40	0	100

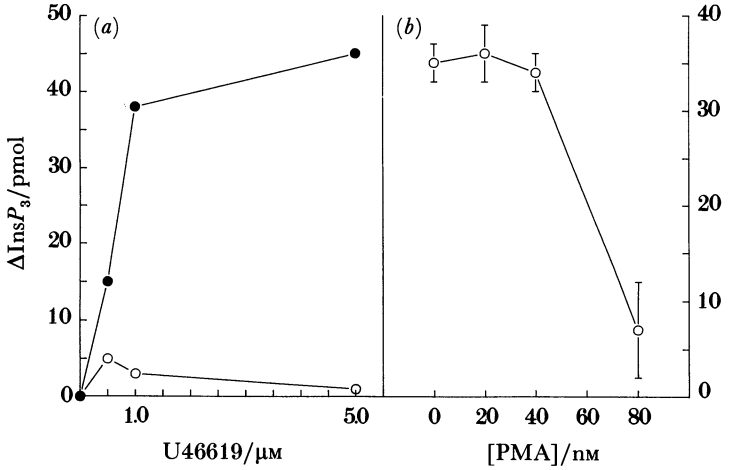

FIGURE 4. Effect of phorbol ester on U46619-induced accumulation of InsP_3 in human platelets. Platelets were incubated for 2 min with (*a*) 80 nM PMA (open circles) or vehicle (filled circles), or (*b*) varied concentrations of PMA before exposure for 15 s to varied concentrations of (*a*) U46619 or (*b*) 2 μM U46619. InsP_3 was assayed and agonist-free control values were subtracted.

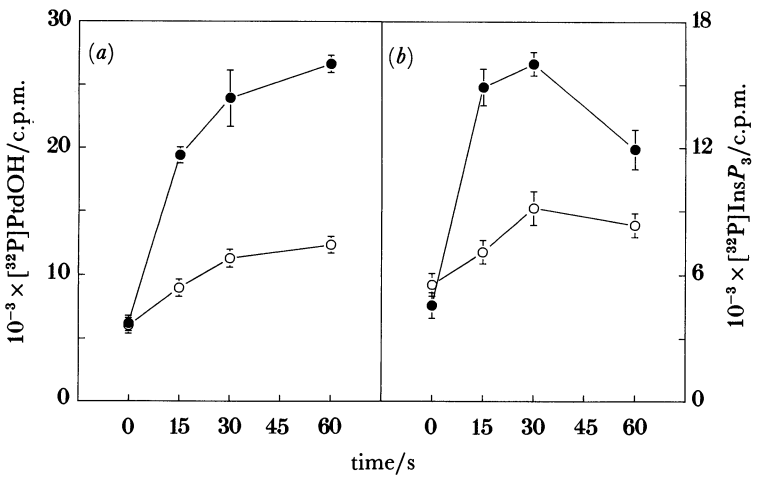

FIGURE 5. Effects of phorbol dibutyrate on the formation of phosphatidic acid and inositol trisphosphate in platelets responding to ONO11113. [^{32}P]-labelled platelets were incubated for 120 s with PDBu (200 nM) (open circles) or vehicle (filled circles) and then exposed to ONO11113 (50 nM) for a further period. [^{32}P]PtdOH (lipid extract) (*a*) and [^{32}P]InsP_3 (aqueous extract) (*b*) were resolved and quantitated.

higher concentrations of these agonists. We have observed inconsistent effects of sphingosine in reversing the inhibition by PMA, possibly because sphingosine is competitive with phosphatidylserine (Bazzi & Nelsestuen 1987) and PMA treatment of platelets has been reported to alter protein kinase C such that it is no longer dependent upon phosphatidylserine (Tapley & Murray 1985). Interestingly, the inhibitory effects of PDBu on ONO11113-induced PtdOH are greater than those on thrombin-induced PtdOH, whereas such effects on InsP_3 are similar (figure 6).

Interpretation of these results has been rendered more complex by recent observations on Ins(1,4,5)P_3 metabolism. The enzyme that hydrolyses Ins(1,4,5)P_3, 5-phosphomonoesterase (5-PME), can be phosphorylated by C kinase and thereby be made three times as active as a

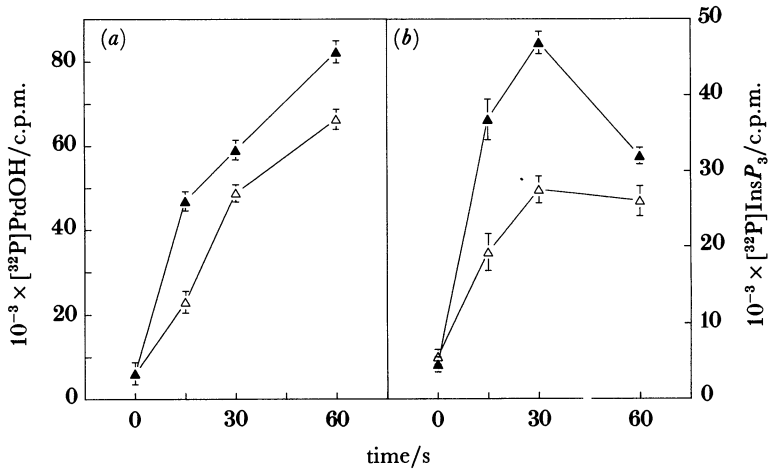

FIGURE 6. Effects of phorbol dibutyrate on the formation of phosphatidic acid (*a*) and inositol trisphosphate (*b*) in platelets responding to thrombin. Platelets were incubated as in figure 5, but for the substitution of α-thrombin (1 U ml^{-1}) for ONO11113.

function of an increased V_{max} (Connolly *et al.* 1986). Agonist-stimulated platelets or platelets exposed to phorbol esters or 1-oleoyl-2-acetyl glycerol (OAG) exhibit enhanced phosphorylation of protein(s) migrating in the 47 kDa region after sodium dodecyl sulphate polyacrylamide gel electrophoresis (SDS–PAGE) and phosphorylated 5-PME travels in this region. In addition, permeabilized platelets incubated with PDBu or OAG and Ins(1,4,5)P_3 hydrolyse Ins(1,4,5)P_3 twice as rapidly as they do in the absence of these C-kinase agonists (Molina y Vedia & Lapetina 1986). None the less, it appears unlikely that 5-PME accounts fully for the inhibitory effect of PMA on InsP_3 accumulation described above, an inhibition that can reach more than 90%. In other tissues, stimulation of 5-PME is clearly an insufficient explanation for the effects of phorbol esters. Membranes derived from 1321 N1 astrocytoma cells that have been incubated with PMA display less phospholipase C activity in response to carbachol, generating less InsP_3 than PMA-free control membranes (Orellana *et al.* 1987). These membranes have no detectable 5-PME. Clearly, it would be desirable to perform comparable experiments with platelet membranes.

Possible mechanisms of inhibition

What, then, are the possible explanations for the inhibitory effects observed? We have examined whether other secondary phenomena, such as cytoplasmic alkalinization, depression of cytoplasmic Ca^{2+} levels, or generation of cyclic AMP or cyclic GMP, might be responsible for the decreased accumulation of InsP_3 and PtdOH in platelets that have been challenged with thromboxane A_2 mimetics after exposure to phorbol esters. Dimethylamiloride, which blocks the Na^+–H^+ antiporter (Besterman *et al.* 1985), or the divalent cationophore A23187 are unable to reverse the effects of PMA on PtdOH levels in response to U46619 (table 2). Further, weak bases, such as NH_4Cl, that diffuse across membranes in their uncharged form and, on protonation, elevate compartmental pH, cannot duplicate the effect of PMA, nor can proton ionophores, such as monensin, that dissipate extracellular–intracellular pH gradients (table 2). Cyclic AMP, which inhibits the activation of phospholipase C in platelets (Rittenhouse-Simmons 1979) is not elevated in response to PMA (figure 7, shown in comparison with

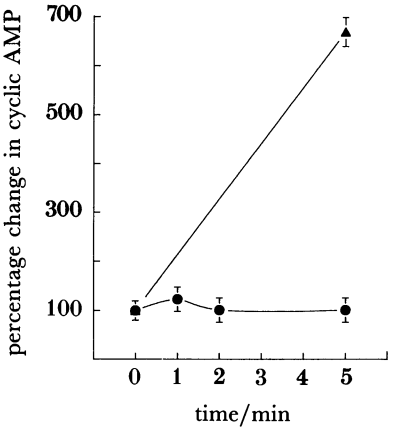

FIGURE 7. Effects of PMA or PGE_1 on cyclic AMP accumulation in platelets. Platelets (7.5×10^8) were incubated for various periods with PMA (160 nM) (circles), vehicle, or (for 5 min) prostaglandin E_1 (1 μM) (triangle). Levels of cyclic AMP in TCA-soluble extracts were determined after radio-immunoassay.

TABLE 2. FAILURE OF VARIOUS AGENTS TO MIMIC OR BLOCK THE INHIBITION BY PMA OF PTDOH ACCUMULATION IN PLATELETS

(Aspirin-treated, [^{32}P]-labelled platelets were exposed to 2 μM U46619 (a thromboxane A_2/prostaglandin H_2 analogue) for 60 s with or without prior incubation for 2 min with 60 nM phorbol myristate acetate (PMA), dimethylamiloride (DMA), or the other agents indicated. Formation of [^{32}P]PtdOH was measured and agonist-free control values were subtracted from these values.)

	Δ[^{32}P]PtdOH		percentage inhibition with PMA
additions	U46619 − PMA	U46619 + PMA	
none	10241 ± 1347	5120	50
DMA (10 μM)	10532 ± 832	4898	52
monensin (10 μM)	10960 ± 650	—	
NH_4Cl (0.2–20 mM)	11193 ± 1100	—	
A23187	12215 ± 900	4886	60

PGE_1, an adenylyl cyclase stimulus). Thus, although the activation of protein kinase C has been shown in other cells to potentiate the formation of cyclic AMP (Yoshimasa *et al.* 1987; Rozengurt *et al.* 1987), it is insufficient of itself to elevate levels of cyclic AMP in platelets. Cyclic GMP also inhibits agonist-induced formation of InsP_3 and PtdOH as seen in figure 8, where the inhibitory effects of nitroprusside-stimulated cyclic GMP or 160 nM PMA are compared. The inhibitory effects of both these agents are approximately 30 % reversible by the addition of the kinase inhibitor H-7 (100 μM, not shown). PMA does not, however, cause an elevation of cyclic GMP in human platelets (figure 9).

In considering other known targets for protein kinase C, we must focus on the class of GTP-binding–signal-transducing proteins known as 'G proteins'. The earliest and best characterized G protein is G_s, a heterotrimeric (α, β, γ) complex in the membrane that couples a receptor-directed stimulatory signal at the cell surface to adenylyl cyclase. The α subunit of the trimer binds GTP and GDP and is capable of enhanced hydrolysis of the newly-bound GTP in response to a receptor-directed stimulus. In binding GTP, α_s dissociates from β–γ and interacts with and activates adenylyl cyclase. The non-hydrolysable GTP analogue, GTPγS, is even more effective in causing this activation, whereas GDPβS tends to be inhibitory. By altering

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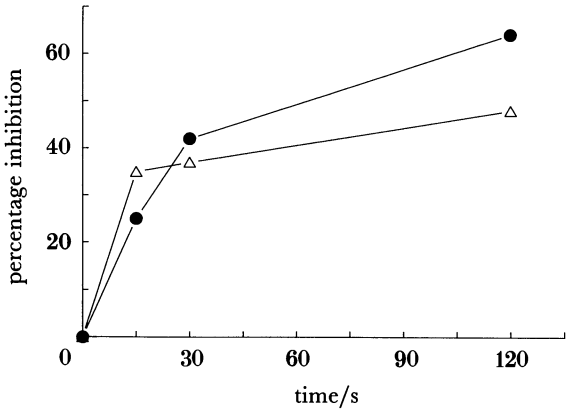

FIGURE 8. Inhibition by phorbol ester or sodium nitroprusside of phosphatidic acid accumulation in U46619-stimulated platelets. [^{32}P]-labelled platelets were incubated for different periods with buffer, NaNP (5 μM, open triangles) or PMA (160 nM, filled circles) before the addition of buffer or U46619 (2 μM) for 60 s. Formation of [^{32}P]PtdOH was quantified and percentage inhibition determined.

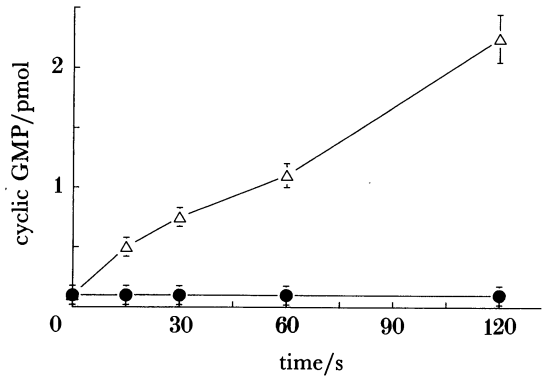

FIGURE 9. Effects of PMA or sodium nitroprusside on cyclic GMP levels in platelets. Platelets (1.6×10^8) were incubated for varied periods with NaNP (5 μM, open triangles) or PMA (160 nM, filled circles). Cyclic GMP in TCA-soluble extracts was determined after radio-immunoassay.

G_s, one can perturb the transmission of a modulatory signal that would culminate in the generation of cyclic AMP (see Rodbell 1980). Inhibitory receptors for adenylyl cyclase also exist and are coupled to this enzyme through a different G protein, G_i. Evidence that G_i is different from G_s came from experiments by Ui (1984) in which a toxin isolated from the culture media of *Bordetella pertussis* was found to suppress the inhibitory effects of α_2-adrenergic agonists on adenylyl cyclase. Pertussis toxin (PT) is a multimer consisting of an NAD-utilizing, ADP-ribosylating monomer (A or S_1) and a cell-binding oligomer (B). Entry of S_1 into a target cell, with the aid of B, allows S_1 to ADP-ribosylate a GTP-binding subunit different from α_s. This α_i species is a 41 kDa protein that is optimally ADP-ribosylated on a susceptible cysteine residue when in association with β–γ. ADP-ribosylation of α_i renders G_i incapable of transmitting an inhibitory signal from a receptor to adenylyl cyclase. Platelets contain a G_i that couples α_2-adrenergic receptors to adenylyl cyclase (Jakobs *et al.* 1985*a*), and α-thrombin, platelet-activating factor, and epinephrine inhibit adenylyl cyclase in platelet membranes in a manner that is blocked by pertussis toxin (Houslay *et al.* 1986*b*). Moreover, treatment of platelets with PMA impairs the GTP-dependent and hormone-induced inhibition of adenylyl

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cyclase and phosphorylates α_i (Jakobs *et al.* 1985*b*; Katada *et al.* 1985). Thus, an important target for protein kinase C appears to be G_i, but other G proteins, and possibly receptors and associated enzymes (see, for example, Yoshimasa *et al.* 1987), may be acted upon as well. When we regard the pattern of phosphorylated platelet protein bands on SDS–PAGE gels before and after exposure to PMA, we can see numerous sites of intensified signal associated with PMA treatment, apart from the intense 47 kDa band and the fainter band at 41 kDa. One of these other bands may hold the key to the effects of C kinase on phospholipase C.

G proteins and phospholipase C

Activation of phospholipase C appears to be dependent upon a G protein or G proteins. Haslam & Davidson (1984) have shown that voltage-permeabilized platelets exposed to GTPγS generate diacylglycerol, an effect that is blocked by GDPβS. GTP potentiates the stimulatory effects of thrombin under these conditions. In saponin-permeabilized platelets, GDPβS blocks the accumulations of InsP_3 and diacylglycerol that occur in response to α- or γ-thrombin (Brass *et al.* 1986) as well as the Ca^{2+} flux and phosphatidic acid that accumulates in response to U46619 (Brass *et al.* 1987). We have also examined the effects of pertussis toxin (PT) and S_1 on such activation, and the results are instructive about both the role of G_i and the pitfalls to be encountered in utilizing pertussis toxin. PT alone can activate phospholipase C, as monitored by accumulation of InsP_3 and PtdOH (table 3) (Banga *et al.* 1987). That this

TABLE 3. ACTIVATION OF PLATELET PHOSPHOLIPASE C BY PERTUSSIS HOLOTOXIN

(Aspirin-treated platelets loaded with ^{32}P were incubated with varied concentrations of pertussis toxin for 2 min, and ^{32}P in InsP_3 and PtdOH were quantitated. Values for toxin-free controls were subtracted (Banga *et al.* 1987).)

toxin	Δ[^{32}P] c.p.m.	
μg ml^{-1}	InsP_3	PtdOH
5	2900	1500
10	5500	4500
15	11500	11600
20	13500	12400

activation is unrelated to the ADP-ribosylating activity of the toxin is revealed by the facts that (i) PT whose ADP-ribosylating activity has been eliminated by reaction with *N*-ethylmaleimide can still activate platelets; and (ii) the S_1 monomer of PT, when separated from the remainder of the toxin, is unable to activate platelets, despite its ability to modify G_i. This is observable with intact or saponin-permeabilized platelets (table 4). Thus, the use of S_1 enables us to sidestep the confounding effects of another agonist in our studies of thrombin or ONO11113-activated platelets. α-Thrombin is able both to activate G_i (and thereby inhibit adenylyl cyclase) and, as described above, to activate phospholipase C. When S_1 is added to saponin-permeabilized platelets and ADP-ribosylation and accumulations of PtdOH and cyclic AMP are measured with [^{32}P]-labelled precursors, the G-proteins modulating adenylyl cyclase and phospholipase C can be differentiated. Under conditions in which significant inhibition of α-thrombin's effects on adenylyl cyclase (G_i) is expressed, we see negligible inhibition of α-thrombin-activated phospholipase C (table 5). The α-thrombin-coupled G protein that regulates phospholipase C can thus be distinguished from that which negatively modulates

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Table 4. Ability of pertussis toxin and derivatives to activate phospholipase C and ADP-ribosylate 41 kDa platelet protein

(Aspirin-treated platelets were incubated with (+) or without (−) permeabilizing saponin and buffer (control), or pertussis toxin (PT, 20 μg ml^{-1}), or *N*-ethylmaleimide (NEM)-treated PT (20 μg ml^{-1}), or the ADP-ribosylating subunit of PT (PT–S_1, 6 μg ml^{-1}) for 2 min in the presence of [γ-^{32}P]ATP (+). [^{32}P]PtdOH was quantified. When platelets were incubated under non-permeabilizing conditions (−), [^{32}P]-labelled platelets were used and [^{32}P]PtdOH quantified.

For determination of ADP-ribosylation, permeabilized platelets (+) were incubated with [^{32}P]NAD and buffer (control), or PT, or PT–NEM, or PT–S_1 (Banga *et al.* 1987).

All values are given as a percentage of the control; ND, not determined.)

	PtdOH		ADP-ribosylation
	−	+	+
PT	280	200	280
PT–NEM	300	ND	100
PT–S_1	100	100	350

Table 5. Differential effect of PT/S_1 in interfering with thrombin modulation of adenylyl cyclase and phospholipase C

(Aspirin-treated platelets were incubated with saponin and 0–30 μg ml^{-1} pertussis toxin S_1 monomer (PT/S_1) and [^{32}P]NAD for 10 min. Maximum ADP-ribosylation was achieved with 10 μg ml^{-1} PT/S_1 for 60 min, as determined by ^{32}P incorporation into a 41 kDa band measured after SDS–PAGE. In parallel experiments, permeabilized platelets were incubated with 0–30 μg ml^{-1} PT/S_1 and (i) buffer, or 0.5 U ml^{-1} α-thrombin, and PGE_1 (0.5 μM) + [α-^{32}P]ATP for determination of cyclic AMP; or (ii) buffer, or 0.5 U ml^{-1} α-thrombin, + [γ-^{32}P]ATP for determination of PtdOH after 2.5 min. Ordinarily, thrombin *suppresses* the PGE_1-stimulated accumulation of cyclic AMP. PT/S_1 interferes with that suppression, resulting in more accumulation of cyclic AMP. Thus, the percentage inhibition given here is not inhibition of adenylyl cyclase but inhibition of the effects of thrombin in inhibiting adenylyl cyclase (Banga *et al.* 1988).)

percentage of max. ADP-ribosylation	percentage inhibition adenylyl cyclase	phospholipase C
25	25 ± 3	0
35	33 ± 2	3 ± 2
50	38 ± 4	10 ± 3

adenylyl cyclase. S_1 does not alter the effects of ONO11113 on phospholipase C either, and thromboxane receptor-directed agonists are known not to inhibit adenylyl cyclase (Houslay *et al.* 1986; Brass *et al.* 1987).

An array of platelet agonists, including α- and γ-thrombin, and thromboxane A_2 analogues appear to affect a G protein, or G proteins, coupled to phospholipase C. In contrast, α_2-adrenergic agonists do not activate phospholipase C (Banga *et al.* 1986; Sweatt *et al.* 1986; Siess *et al.* 1984) although they activate G_i. α-Thrombin, alone, is a potent agonist for both phospholipase C and G_i. These separable effects point to the possible existence of more than one G protein, of which the G protein coupled to phospholipase C can be designated 'G_p'. It would also seem possible, therefore, that such a 'G_p' could provide another target for protein kinase C in inhibiting phospholipase C, independently of any involvement of G_i. As yet, however, G_p has not been isolated and identified as a phosphorylated species in platelets exposed to PMA or agonists. It may prove to be quite similar to G_i, but lacking, for example, a cysteine susceptible to ADP-ribosylation by S_1. Such a G_p (i.e. its α_p) might none the less be quite susceptible to phosphorylation. This possibility obviously requires much closer scrutiny.

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Conclusions

An illustrative compilation of the known effects, to date, of protein kinase C on platelet metabolic responses is presented in figure 10. There are at least two additional possible sites for protein kinase C action indicated, and the existence of even more targets would not be surprising. Yet the obvious question remains of what the effects of PMA and other exogenous stimuli for protein kinase C have to do with endogenous regulatory mechanisms. Experiments

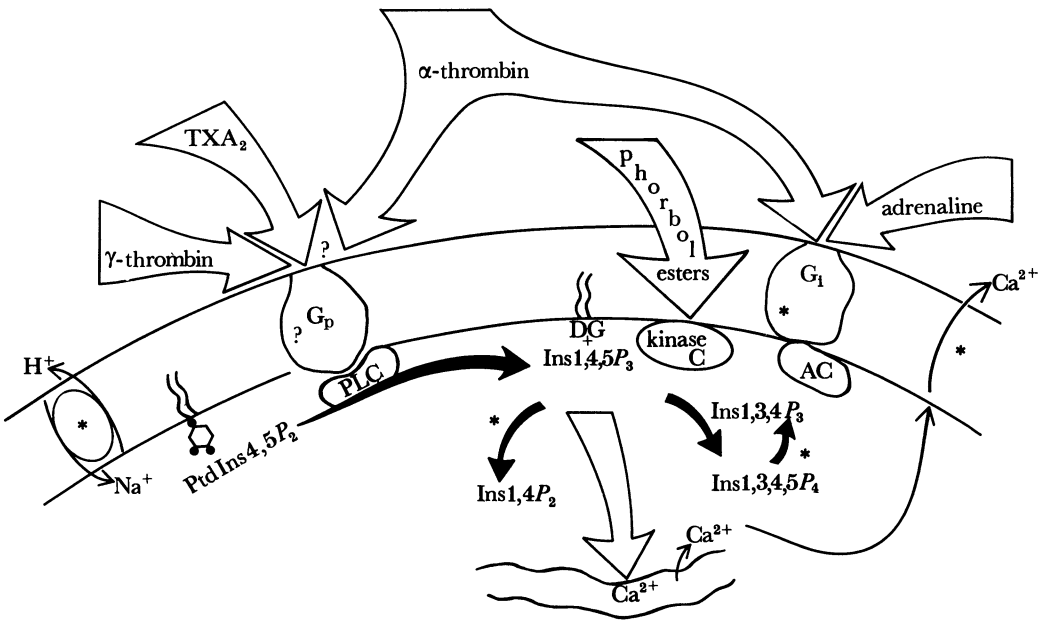

Figure 10. Summary of routes by which platelets are activated and inhibited. This scheme is not meant to imply that two different, physically separate binding sites for α-thrombin or γ-thrombin exist, merely that the site is variously coupled. Further, adenylyl cyclase, the protein, is considered to span the plasma membrane. The catalytic activity (AC) is at the cytoplasmic face. Asterisks indicate known targets of protein kinase C-directed phorbol esters; ?, targets putatively acted upon by C kinase. G_i is the known target of persussis toxin-derived S_1 ADP-ribosylating activity.

examining the effects of down-modulation of protein kinase C on Swiss-mouse 3T3 cells indicate that such cells have higher basal levels of inositol phosphates and respond more dramatically to bombesin with accumulation of inositol phosphates (Brown *et al.* 1987). Although such down-modulation by extended exposure to PMA is not feasible for platelets, sphingosine might be employed for similar ends. Finally, if feed-back inhibition by protein kinase C is to be relevant to agonist-stimulated platelets, then a mechanism must be defined that accounts for homologous desensitization. A plenipotent mechanism affecting 5-PME and phospholipase C does not meet this criterion. Thus, the endogenous mechanism must be directed more finely (probably in a receptor-dependent way) than the mechanism utilized by PMA. The experimental elucidation of this endogenous mechanism is an important direction for future investigation.

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