Biochem. J. (1991) 278, 475–480 (Printed in Great Britain)

Protein kinase C regulates the stimulated accumulation of 3-phosphorylated phosphoinositides in platelets

Warren G. KING,* Gregory L. KUCERA,* Alexander SORISKY,† Jin ZHANG*
and Susan E. RITTENHOUSE*‡
*Department of Biochemistry and †Metabolic Unit, University of Vermont College of Medicine, Burlington, VT 05405, U.S.A.

We have shown that platelets stimulated with thrombin or guanosine 5′-[γ-thio]triphosphate (GTP[S]), both of which activate phospholipase C and protein kinase C (PKC), show enhancement of 3-phosphorylated phosphoinositide accumulation (3-PPI). We now report the following. (1) Inhibition of thrombin- or GTP[S]-stimulated PKC by pseudosubstrate peptide (RFARK) added to permeabilized platelets markedly inhibits 3-PPI, whereas the serine/threonine phosphatase inhibitor, okadaic acid, promotes 3-PPI. PKC activity, insufficient in itself for fully activating 3-PPI, appears crucial to receptor and post-receptor stimulation of 3-PPI, even when tyrosine phosphorylation is unimpaired. (2) Alteration of G_i by ADP-ribosylation only slightly affects the stimulation of 3-PPI by thrombin, and activation of the G-protein G_i by adrenaline has no effect on 3-PPI. (3) Inhibition of PKC blocks activated secretion of platelet-derived growth factor (PDGF). However, PDGF cannot promote platelet 3-PPI, and thus cannot account for the inhibitory effects of RFARK on 3-PPI.

INTRODUCTION

Accumulation of phosphoinositides phosphorylated at the D-3 position of the inositide ring has now been described for a variety of nucleated and anucleate cells exposed to appropriate agonists [1–8]. The 3-phosphorylated phosphoinositides have been postulated to be involved in mitogenic signalling responses in some nucleated cells, and PtdIns 3-kinase is associated with activated tyrosine-phosphorylated platelet-derived growth factor (PDGF) receptors [9] and middle T/pp60$^{c\text{-}src}$ complexes [10].

Although the mechanism of action of such novel phosphoinositides has not yet been demonstrated, the evidence that they perform an important signalling function in many cell types is compelling [11,12]. It is therefore of interest to elucidate the factors regulating 3-phosphorylated phosphoinositide accumulation (3-PPI). The anucleate terminally differentiated platelet offers a useful model system for examining 3-PPI, uncomplicated by variables such as protein synthesis. We have reported recently that human platelet 3-PPI appears to be controlled by GTP-binding protein(s), inasmuch as guanosine 5′-[γ-thio]triphosphate (GTP[S]) [1,2], but not guanosine 5′-[β-thio]diphosphate, is stimulatory. Both thrombin (a mitogenic agent in other systems) and GTP[S] activate phospholipase C (PLC) and consequently protein kinase C (PKC). Although direct stimulation of PKC by tumour-promoting phorbol esters can promote 3-PPI, such accumulation is not as dramatic or as specific as that achieved in platelets exposed to thrombin or GTP[S] [1,2]. This nonetheless leaves in question the possible regulatory function of PLC activation and consequent PKC stimulation, and the nature of modulation by thrombin and G-protein(s). PKC activation could exert an influence at several points along possible routes of generation and/or breakdown of 3-phosphorylated species. Activation of PKC is known to promote secretion by platelets of granule-stored substituents, including PDGF [13], and inhibition of PKC inhibits secretion [14]. If PDGF is capable of promoting 3-PPI in the platelet, then inhibition of granule secretion would impair 3-PPI. Further, activation of PKC has been reported to inhibit thrombin- and adrenaline-sensitive activation of the ADP-ribosylatable GTP-binding protein complex, G_i [15]. Therefore, we have explored several related effects of platelet PKC activation, asking: (1) does inhibition of PKC inhibit 3-PPI at the receptor and/or post-receptor levels? (2) does PKC inhibition exert effects by impairing agonist-induced tyrosine phosphorylation? (3) can PDGF activate 3-PPI, and if so, are the effects of thrombin, GTP[S] and PKC on 3-PPI mediated by PDGF? (4) is ADP-ribosylatable G_i involved in the regulation of 3-PPI?

EXPERIMENTAL

Materials

Except as otherwise indicated, sources of reagents utilized were as described previously [1]. α-Thrombin was given by Dr. Kenneth Mann (University of Vermont, Burlington, VT, U.S.A.). β-Thromboglobulin radioimmunoassay kit was from Amersham Corp. Extractigel was purchased from Pierce. The PKC pseudosubstrate inhibitor RFARK was generously provided by Dr. Susan Jaken (Alton Jones Cell Science Center, Lake Placid, NY, U.S.A.). S_1 monomer was prepared and isolated from pertussis toxin. PDGF (BB homodimer, recombinant c-*sis*) was a gift from Dr. Tom Deuel (Washington University, St. Louis, MO, U.S.A.), and AB heterodimer was from Creative Biomolecules (Hopkinton, MA, U.S.A.). Anti-phosphotyrosine monoclonal antibody (MAb) 4G10 was kindly provided by Dr. Brian Druker (Dana Farber Cancer Institute, Boston, MA, U.S.A.), and ^{125}I and Iodogen were purchased from Amersham and Pierce respectively. Monoclonal antibody (MAb) 327, specific for pp60$^{c\text{-}src}$, was given by Dr. Joan Brugge (University of Pennsylvania, Philadelphia, PA, U.S.A.). PtdIns 3-kinase was generously given by Dr. Lew Cantley and Dr. Chris Carpenter (Tufts University, Boston, MA, U.S.A.).

Preparation of platelets

Human platelet-rich plasma was prepared fresh daily from 1 unit of blood donated by healthy individuals as described pre-

Abbreviations used: 3-PPI, 3-phosphorylated phosphoinositide accumulation; GTP[S], guanosine 5′-[γ-thio]triphosphate; PDBu, β-phorbol dibutyrate; PDGF, platelet-derived growth factor; PKC, protein kinase C; PLC, phospholipase C; PtdOH, phosphatidic acid; RFARK, Arg-Phe-Ala-Arg-Lys-Gly-Ala-Leu-Arg-Gln-Lys-Asn-Val (pseudosubstrate peptide); MAb, monoclonal antibody.

‡ To whom correspondence should be addressed.

viously [1], and were treated with 1 mM-aspirin, washed, and resuspended in Buffer A (132 mM-NaCl, 12 mM-sodium citrate, 5.6 mM-dextrose, 2.0 mM-Hepes, 2.8 mM-KCl, 8.9 mM-$NaHCO_3$, 0.86 mM-$MgCl_2$, 10 mM-Trizma base). When ^{32}P-labelled platelets were used, platelets were labelled for 90 min with [^{32}P]P_i, washed, and suspended in phosphate-free buffer, as presented elsewhere [1].

Incubations with intact platelets

^{32}P-labelled platelets (0.5 ml; 2×10^9/ml in buffer A, pH 7.3) was warmed to 37 °C for 5 min, followed by addition of buffer or agonist such as thrombin (1 unit/ml $\simeq$ 10 nM) or PDGF (up to 200 ng/ml) for 1 min except where otherwise indicated. The reactions were quenched with chloroform/methanol/1 M-HCl (4:10:1, by vol.) and the lipids extracted as described previously [1,16], being either resolved by t.l.c., eluted and deacylated [1,10], or deacylated directly, before h.p.l.c. In other studies, platelets were warmed to 37 °C and then added to tubes containing buffer, vehicle (0.25% dimethyl sulphoxide), β-phorbol dibutyrate (PDBu; 200 nM) + ionophore A23187 (1 μM), adrenaline (100 μM) or thrombin (2 units/ml), or combinations of these agents. Samples were removed to SDS/reducing gel buffer at 60 s, and remaining incubations were terminated after 90 s as above.

Incubations with permeabilized platelets

Unlabelled platelet suspensions were adjusted to 1×10^{10}/ml in Buffer A, pH 7.1. As described previously [1], 100 μl of platelets was added to 400 μl of 37 °C permeabilization buffer, containing 120 mM-KCl, 4 mM-$MgCl_2$, 25 mM-NaCl, 1 mM-NaH_2PO_4, 1 mM-EGTA, 269 μM-$CaCl_2$, 0.625 mM-[γ-^{32}P]ATP (40 mCi/mmol), 31 μg of saponin/ml, 15 mM-Hepes, pH 7.1 (Buffer B; [17]). The amount of total Ca^{2+} added was adjusted to ensure free [Ca^{2+}] of 0.1 μM [18]. At 1 min after the addition to Buffer B, thrombin (2 units/ml), okadaic acid (1 μM) or GTP[S] (10 μM) was added. The reactions were quenched 5 min after agonist addition as described in [1]. When RFARK was used, it was present (5–25 μM) at the time of addition of permeabilization buffer.

Incubations with soluble fractions

Platelets were lysed with ice-cold buffer containing Triton X-100, yielding a final concentration of 1% Triton X-100, 5 mM-EGTA, 0.2 mM-leupeptin and 1.0 mM-sodium vanadate, pH 7.4. Lysates were centrifuged at 100000 *g* for 3 h at 4 °C, and supernatants were passed through Extractigel to remove inhibitory Triton. Eluates were incubated for 2 min at 37 °C in the presence of 50 μM-ATP, 10 μCi of [γ-^{32}P]ATP, 5 mM-$MgCl_2$, 100 mM-NaCl, 20 mM-Tris/HCl, 200 μg of PtdIns or PtdIns(4)*P*/ml at pH 7.3, with or without 25 μM-RFARK. Portions were resolved on SDS-reducing gels (7.5% acrylamide) by PAGE [16] and ^{32}P was quantified. Lipids were extracted and deacylated before resolution of glycerophospholipids, described below and in [1]. As a direct test of RFARK effects on PtdIns 3-kinase, a highly purified preparation of liver PtdIns 3-kinase was incubated in the presence or absence of 25 μM-RFARK according to the assay protocol previously described [19], and formation of PtdIns(3)*P* was determined.

ADP-ribosylation of G_i by S_1

S_1, the ADP-ribosylating enzymic subunit of pertussis toxin, was prepared [20,21]. In four experiments run in duplicate, platelets (100 μl of 1×10^{10}/ml) in Buffer A, pH 7.1 at 37 °C, were incubated with 400 μl of Buffer B containing 0.5 mM-NAD^+ and 20 μg of S_1/ml or buffer for 9 min, followed by addition of 25 μCi of [γ-^{32}P]ATP/ml for an additional 1 min. At 10 min, buffer or thrombin (1 unit/ml) was added and the reaction was allowed to proceed for an additional 5 min. The reaction was quenched and the lipids were resolved as for other samples.

Cyclic AMP formation

Cyclic AMP was quantified in the same platelet preparations, by using [α-^{32}P]ATP and the method described [21], based on the ability of prostaglandin E_1 to increase platelet cyclic AMP and of thrombin to counteract that effect.

H.p.l.c. of glycerophospholipids

Glycerophosphoinositol (GroPIns) polyphosphates were separated on a Whatman Partisphere SAX column [1]. This procedure also allowed for the quantification of Gro*P* (R_t = 24 min), as the deacylated form of phosphatidic acid (PtdOH), an indicator of PLC activity. Quantification of Gro*P* was equivalent to that obtained by t.l.c. resolution of [^{32}P]PtdOH [17]. Furthermore, h.p.l.c. analysis of deacylated t.l.c.-resolved [^{32}P]PtdOH showed a peak of radioactivity equal to an R_t of 24 min.

Protein phosphorylation

Phosphorylation of platelet protein p47 was used as a monitor of PKC activity [16]. Just before quenching the reactions as above, samples equal to 5×10^7 platelets were removed and added to an equal volume of 2 × concentrated sample buffer [22], boiled, and the proteins were separated on an SDS/11%-polyacrylamide gel and counted for radioactivity as described [16].

Secretion of α-granule marker

PDGF is contained in α-granules. A 2 ml portion of platelets in Buffer A, pH 7.3, was treated with thrombin (0.1 or 2 units/ml) for 1 min. The reaction was quenched by placing in an ice/water bath, followed by centrifugation at 1200 *g* for 15 min. Then 1 ml of the supernatant was removed and assayed for the α-granule monitor β-thromboglobulin, by the method described in the radioimmunoassay kit.

Immunoprecipitation of pp60$^{c\text{-}src}$ and analysis of tyrosine phosphorylation

Washed unlabelled platelets (6–7 ml of 5×10^8/ml) were mixed with an equal volume of 137 mM-NaCl/2.7 mM-KCl/1 mM-$MgCl_2$/2 mM-$CaCl_2$/2 mM-Na_3VO_4/2% Nonidet P-40/20% glycerol/40 mM-Tris (pH 8.0), containing 0.2 mM-leupeptin, 2% aprotinin and 2 mM-phenylmethanesulphonyl fluoride at 4 °C for 20 min. Insoluble material was removed by centrifugation at 10000 *g* for 20 min at 4 °C. To the supernatant was added 0.05 vol. of 2% (w/v) BSA. To six tubes, each containing 1600 μl of soluble extract, were added 6 μl of MAb 327. Additional tubes received no MAb 327 or no platelet lysate. After 45 min, samples were mixed with Protein A immunadsorbent containing 1 μg of rabbit anti-mouse IgG/sample and immunoprecipitated as described [23]. Washed platelet immunoprecipitates (+MAb 327) were pooled and then distributed equally among 12 tubes. Assays of pp60$^{c\text{-}src}$ tyrosine autophosphorylation and phosphorylation of acid-treated enolase were performed with [γ-^{32}P]ATP as described by Golden *et al.* [24], but in the absence or presence of RFARK, staurosporine or buffer vehicle. Enolase and 60 kDa bands were excised and counted for radioactivity by scintillation spectrophotometry. No activity was observed in the absence of platelet extract or MAb 327.

Immunoblotting of phosphotyrosine

Platelets were exposed to saponin-containing or saponin-free buffer as above, but in the presence of unlabelled ATP. After 60 s, platelets were exposed to 40 μM-LiCl, 25 μM-RFARK/LiCl, 10 μM-Li_4GTP[S], 10 μM-Li_4GTP[S]/25 μM-RFARK, or 2 units of thrombin/ml + LiCl for an additional 60 s or 300 s. Portions

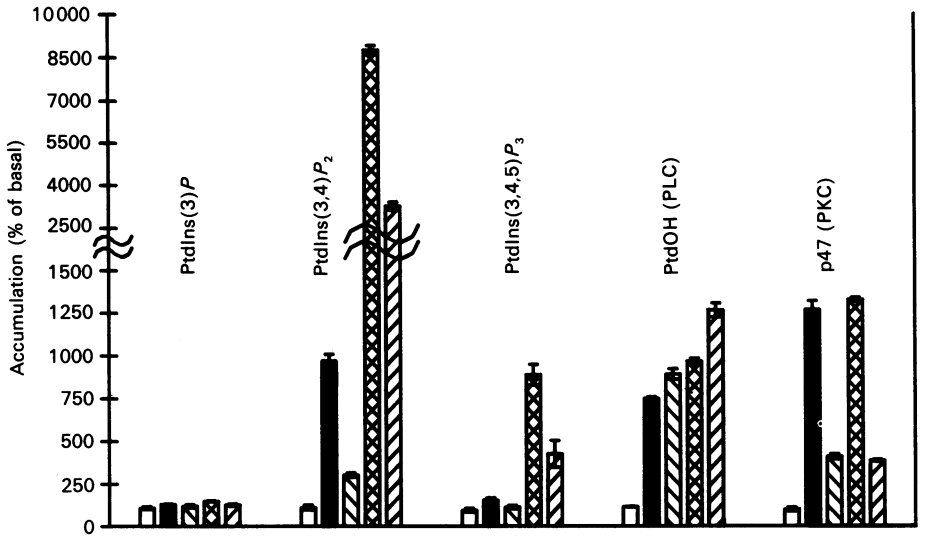

Fig. 1. Effects of PKC inhibition on ^{32}P-labelled 3-PPI, PtdOH and p47

Incubations were carried out with permeabilized platelets as described in the Experimental section. Data are represented as percentages of buffer control. Values represent means ± S.D. (n = 3). In one such experiment, basal phosphoinositides (c.p.m.) were: PtdIns(3)P, 573 ± 5; PtdIns(4)P, 162084 ± 1502; PtdIns(3,4)P_2, 213 ± 36; PtdIns(4,5)P_2, 87326 ± 2644; PtdIns(3,4,5)P_3, 185 ± 53. Key: □, 25 μM-RFARK; ■, thrombin; ▧, thrombin + RFARK; ▩, GTP[S]; ◪, GTP[S] + RFARK; p47, a protein known to be phosphorylated by PKC.

were mixed as above with sample buffer and proteins resolved as above. Proteins from SDS/PAGE were transferred electrophoretically to nitrocellulose filters for 4 h at 4 °C, blocked with 2% gelatin, washed, and incubated overnight with a 1:10000 dilution of MAb 4G10 (stock = 7 mg/ml). ^{125}I-Protein A was prepared by the Iodogen procedure. Blots were washed four times and incubated overnight with ^{125}I-Protein A (10^7 c.p.m./blot). Blots were then washed, and labelled proteins were detected by autoradiography. Radioactivity was quantified with a γ-radiation counter and was linear with platelet number.

RESULTS AND DISCUSSION

Effect of inhibition of PKC on 3-PPI activated by thrombin or GTP[S]

PKC appears to be a major positive regulator of 3-PPI. We have monitored PKC activation by measuring changes in phosphorylation of a 47 kDa protein (p47) that is known to be a substrate for PKC [16,25]. The fact that we can observe stimulation of 3-PPI by either thrombin or GTP[S] in saponin-permeabilized platelets has permitted us to use a highly specific inhibitor of serine/threonine-directed PKC, the pseudosubstrate RFARKGALRQKNV ('RFARK'). This pseudosubstrate corresponds to the N-terminal PKC regulatory domain 19–31 [26,27]. As observed previously [1], stimulation by thrombin of accumulation of PtdIns(3)P is relatively minor, especially in comparison with accumulations of PtdIns(3,4)P_2 in response to this agonist (Fig. 1). RFARK inhibits accumulations of both PtdIns(3,4)P_2 and PtdIns(3,4,5)P_3 in response to thrombin by 77% and 75% respectively, under conditions in which p47 phosphorylation is inhibited by 74%. In contrast, RFARK does not inhibit PLC, as gauged by measurements of PtdOH (which is formed by phosphorylation of diacylglycerol, which is in turn generated uniquely by PLC acting on platelet phosphoinositides [28]), but is in fact stimulatory. This latter observation is consistent with previous reports that PKC acts as a negative regulator of PLC [7,29–31], and also indicates that RFARK is not somehow impairing the function of thrombin. Under the same conditions, RFARK does not significantly alter levels of potential precursors [PtdIns(4)P and PtdIns(4,5)P_2; results not shown]. GTP[S] is more effective than thrombin in stimulating 3-PPI, but increases in PtdIns(3)P remain small. RFARK dose-dependently inhibits the activation of PKC by GTP[S] and impairs accumulations of PtdIns(3,4)P_2 and PtdIns(3,4,5)P_3 in parallel (Fig. 2). At 25 μM, RFARK inhibits accumulation of p47, PtdIns(3,4)P_2 and PtdIns(3,4,5)P_3 by up to 70%, while enhancing PtdOH by 22%.

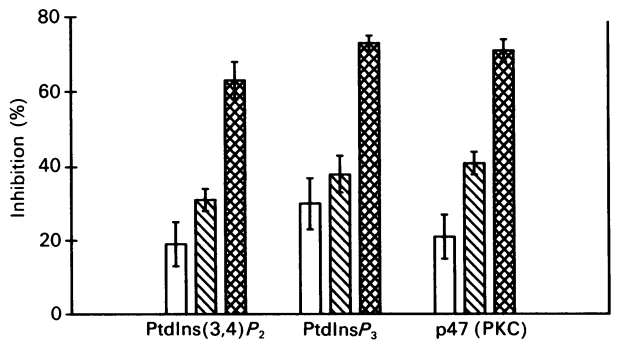

Fig. 2. Parallel effects of RFARK on GTP[S]-stimulated 3-PPI and PKC

Incubations were carried out as in Fig. 1 with varied concentrations of RFARK (□, 5 μM; ▧, 10 μM; ▩, 25 μM). Inhibitions by RFARK of GTP[S]-stimulated increases were calculated. Results are representative of an experiment in duplicate.

Related studies with okadaic acid similarly indicate a role for serine/threonine phosphorylation in 3-PPI. Okadaic acid inhibits protein phosphatases 1 and 2A, two of the four major mammalian protein phosphatases that act at serine/threonine, without affecting protein kinases or tyrosine phosphatase [32]. Like β-phorbol esters, okadaic acid promotes tumour growth. We have observed that 1 μM-okadaic acid, added to saponin-permeabilized platelets, enhances the phosphorylation of six proteins 2–5-fold (including p47) and increases PtdIns(3,4)P_2 1.80 ± 0.26-fold, but has essentially no effect on PtdIns(4)P and PtdIns(4,5)P_2.

In other experiments with Triton-soluble fractions from platelets, we have found that 25 μM-RFARK inhibits the phosphorylation of ten platelet proteins (including p47) by 25–50 %, as well as inhibiting PtdIns 3-kinase (36 ± 4 %) and PtdIns(4)*P* 3-kinase (27 ± 3 %) product accumulation, whereas accumulation of PtdIns 4-kinase- and PtdIns(4)*P* 5-kinase-derived products is unaltered (−5 ± 2 % and 0 ± 3 % respectively). In contrast, highly purified PtdIns 3-kinase, containing no PKC, is unaffected by 25 μM-RFARK. Thus the remote possibility that RFARK directly inhibits PtdIns 3-kinase activity can be ruled out.

Since our studies involve permeabilized platelets, for which [Ca^{2+}] is buffered at 0.1 μM and pH is buffered at 7.1, the rise in cytosolic Ca^{2+} and pH seen in activated intact platelets is not crucial to the 3-PPI response. Thus it should be noted that the effects of PKC inhibition are not exerted via Ca^{2+} or pH changes [33]. Finally, as reported previously [1], although addition of PDBu with or without Ca^{2+} ionophore in intact platelets can stimulate PKC comparably with levels achieved with thrombin, activation of PKC or inhibition of serine/threonine phosphatase is not sufficient to promote 3-PPI [especially PtdIns(3,4)P_2 accumulation] maximally. We can conclude from studies with RFARK (Figs. 1 and 2), however, that PKC activity appears necessary, if not sufficient, for stimulated 3-PPI. Further, although PKC may affect thrombin 'receptor' coupling to a G-protein, PKC also clearly affects G-protein-dependent events at post-receptor site(s), since inhibition of PKC impairs 3-PPI in response to GTP[S].

Effect of inhibition of PKC on tyrosine phosphorylation

Activation of platelets by thrombin leads to increased phosphorylation of several proteins at tyrosine residues ([24,34]; Fig. 3, lanes G and H). The tyrosine kinase $pp60^{c\text{-}src}$ is abundant in platelets [24,34]. We have ascertained directly whether RFARK alters $pp60^{c\text{-}src}$ tyrosine kinase activity by immunoprecipitating $pp60^{c\text{-}src}$ from platelets and examining both autophosphorylation and phosphorylation of enolase (an accepted substrate *in vitro*) in the presence and absence of RFARK. The maximum concentration of RFARK that is added to permeabilized platelets in the studies above inhibits autophosphorylation and enolase phosphorylation by only 1 % and 24 % respectively. This is consistent with its action as a specific PKC inhibitor. In contrast, we have observed that another potent PKC inhibitor, staurosporine, completely inhibits $pp60^{c\text{-}src}$ autophosphorylation and enolase phosphorylation at concentrations (1–10 μM) that are necessary to inhibit platelet PKC (results not shown). Moreover, RFARK does not alter tyrosine phosphorylation in permeabilized platelets (Fig. 3), as detected by phosphotyrosine-specific MAb 4G10. The inhibition of 3-PPI observed during inhibition of PKC by RFARK is achieved in the setting of intact tyrosine phosphorylation. Tyrosine phosphorylation increases in permeabilized, as opposed to intact, platelets with time (60 s versus 5 min; results not shown). However, GTP[S] or thrombin does not cause consistent changes in the phosphotyrosine content of such platelets (apart from a band seen at about 80 kDa with GTP[S]), and no effects of RFARK are seen (Fig. 3). In contrast, intact platelets under the same conditions (no saponin) show conspicuous increases in phosphotyrosine with thrombin (Fig. 3). Thus, although tyrosine phosphorylation may play a role in regulating the activation of 3-PPI, PKC has a role distinct from, or in addition to, tyrosine phosphorylation in promoting this effect. Further, tyrosine phosphorylation observed upon permeabilization of platelets is insufficient to activate 3-PPI.

Effect of PDGF on 3-PPI

The stimulation of 3-PPI achieved in response to PDGF is negligible in comparison with that achieved with thrombin, and

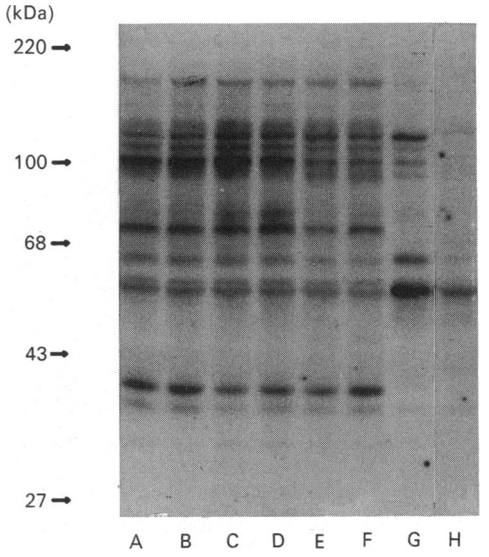

Fig. 3. Effect of platelet agonists and RFARK on tyrosine phosphorylation in platelets

Platelets were incubated in saponin-containing (A–F) or saponin-free (G, H) incubation buffer, as described, for 5 min. Treatment: A, buffer; B, 25 μM-RFARK; C, 10 μM-GTP[S]; D, RFARK/GTP[S]; E, 1 unit of thrombin/ml; F, RFARK/thrombin; G, thrombin; H, buffer. Tyrosine phosphorylation was measured by immunoblotting (shown is ^{125}I-Protein A autoradiogram) and increased with time of incubation for all permeabilized platelet samples (results not shown).

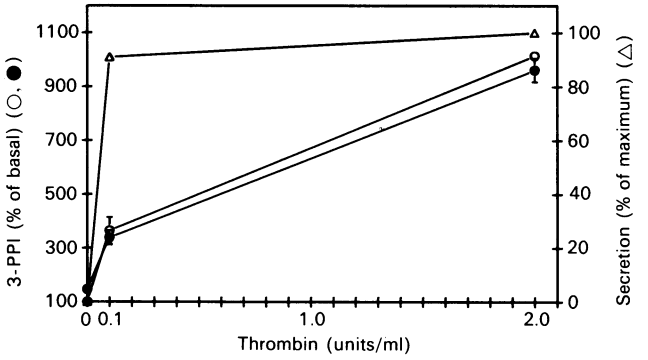

Fig. 4. Effect of PDGF on 3-PPI accumulation

Results for PDGF-AB and PDGF-BB were averaged, since they were not different. Symbols: ○, varied thrombin; ●, PDGF + varied thrombin; △, secretion of PDGF-containing α-granules in response to thrombin. Results are averages ± ranges ($n = 2$).

cannot account for this effect of thrombin. PDGF was one of the first agents reported to stimulate PtdIns 3-kinase product formation in fibroblasts [9]. We have tested whether PDGF (which is secreted from α-granule stores in stimulated platelets), at the concentration known to be present with thrombin-stimulated platelets [35], is responsible for the 3-PPI observed in platelets exposed to thrombin or GTP[S]. As shown in Fig. 4, 3-PPI in response to thrombin is still a function of thrombin concentrations, even in a range that yields maximal α-granule secretion. Whether AB- or BB-PDGF is used, little increase in 3-PPI is observed in the absence of thrombin. Finally, PDGF (AB or BB) added to platelets in the presence of different doses of thrombin does not appear to modulate 3-PPI. We can therefore conclude that thrombin is not acting indirectly, via PDGF. Thus, whereas we have observed that RFARK inhibits platelet secretion in response to GTP[S] or PDBu by 82–100 %, in confirmation of

reported findings with other agonists and PKC inhibitors, it is not PDGF secretion that mediates the stimulation of 3-PPI in the platelet.

Effect of inhibition of G_i on 3-PPI

Apparently, ADP-ribosylatable GTP-binding protein (G_i) contributes only partially to regulation of 3-PPI. The catalytic subunit of pertussis toxin, S_1, ADP-ribosylates G_i and uncouples this adenylate cyclase-inhibitory GTP-binding protein from control by thrombin [20,21]. Whereas treatment with S_1 inhibits thrombin's G_i-dependent regulation of adenylate cyclase by $97.9 \pm 3.1\%$ ($\pm$S.D.), it inhibits 3-PPI by only $24.2 \pm 4.4\%$ (S.D.) and PtdOH accumulation (as a monitor of PLC) by $18.6 \pm 4.3\%$ (S.D.). A small contribution of this G-protein to PLC activation has been described previously [21], and it is quite possible that the inhibition observed can be attributed to impairment of PKC by this route. It seems most probable, however, that the G_i that modulates adenylate cyclase is not responsible for the major part of 3-PPI or PLC [21] modulation. This is underscored by the findings that adrenaline, which activates G_i and inhibits adenylate cyclase [36], does not perturb 3-PPI (this study; results not shown) or PLC [37] significantly, or modulate the effects of PDBu, and therefore PKC (results not shown). The G-protein(s) that regulate PLC and/or 3-PPI have not yet been identified. A recent report [38] indicates that a heterotrimeric G-protein, G_z, which is phosphorylated by PKC, is present in platelets. Its function is unknown, but it is not ADP-ribosylated by pertussis toxin/S_1. Possibly G_z, G_q or one of the small-molecular-mass G-proteins in platelets is responsible for the effects of GTP[S] on 3-PPI.

Conclusion

There are several potential targets for PKC in modulating 3-PPI, especially given the involvement of GTP-binding protein(s). These include: agonist/receptor binding, receptor/GTP-binding-protein coupling, modulation of GTPase-activating protein ('GAP') or GDP-release factor ('GRF') [39,40], if a small GTP-binding protein is involved, GTP-binding-protein/enzyme coupling, and/or enzyme activity itself. 'Enzyme' can refer to phosphoinositide 3-kinase, 3-phosphoinositide 3-phosphatase [41], 4-kinase acting on PtdIns(3)*P* [42], and/or possible intermediate regulatory enzyme(s). A direct effect of PKC on 3-kinase enzyme activity is implied by our studies with soluble fractions assayed in the presence of RFARK, although inhibition of 3-PPI here is not as marked as that observed with permeabilized platelets exposed to agonists. Inasmuch as 3-PPI in response to thrombin or GTP[S] is strongly inhibited when PKC activation is blocked, factors affecting GDP release, GTP-binding-protein/enzyme, and/or enzyme activity remain as possible targets for PKC's effects, apart from any receptor effects, and thus as sensitive targets for PKC inhibition. In other studies (J. Zhang, M. Fry, M. Waterfield, S. Jaken, L. Liao, J. E. B. Fox & S. E. Rittenhouse, unpublished work), we have found that PtdIns 3-kinase and PtdIns(4)*P* 3-kinase activities and PKC are co-localized in a sub-fraction derived from thrombin-stimulated platelets. Ultimately, purification of the platelet 3-kinase(s) should allow us to determine whether they are direct substrates for PKC. Nonetheless, the observation that PKC activation is essential to normal accumulation of 3-phosphorylated phosphoinositides in platelets is clearly an important feature of the '3-PPI effect', and may hold true for nucleated mitogenically stimulated cells as well.

Another issue that remains to be addressed is the nature of the missing factor(s) that promotes the pronounced increase in 3-PPI seen with thrombin, thromboxane A_2 analogue, or GTP[S] [1], but not with PDBu. The missing factor(s) may relate, for example, to a localized activation of PKC by the first three agonists, compared with a more diffuse activation achieved with PDBu. Resolution of this issue awaits further studies.

This work was supported by National Institutes of Health Grant HL-38622 (to S. E. R.), a research fellowship of the Medical Research Council of Canada (to A. S.), and Training Grant HL 07594. The blood-drawing services of the General Clinical Research Center (GCRC RR109) of the Medical Center Hospital of Vermont are gratefully acknowledged, as is the help of Lisa McNaney in the preparation of the manuscript. We also extend special thanks to Dr. Joan Brugge for MAb 327 and advice on assaying $pp60^{c\text{-}src}$ tyrosine kinase activity, to Dr. Bill Church (University of Vermont) and Dr. Brian Druker for helpful advice on immunoblotting techniques, and to Dr. Lew Cantley and Dr. Chris Carpenter for providing their PtdIns 3-kinase for assay and useful discussions.

REFERENCES

1. Kucera, G. L. & Rittenhouse, S. E. (1990) J. Biol. Chem. **265**, 5345–5348
2. Kucera, G. L. & Rittenhouse, S. E. (1990) J. Cell. Biochem. Suppl. **14B**, 261
3. Whitman, M., Kaplan, D. R., Roberts, T. & Cantley, L. (1985) Biochem. J. **247**, 165–174
4. Auger, K. R., Serunian, L. A., Soltoff, S. P., Libby, P. & Cantley, L. C. (1989) Cell **57**, 167–175
5. Traynor-Kaplan, A. E., Thompson, B. L., Harris, A. L., Taylor, P., Omann, G. M. & Sklar, L. A. (1989) J. Biol. Chem. **264**, 15668–15673
6. Pignataro, O. P. & Ascoli, M. J. (1990) J. Biol. Chem. **265**, 1718–1723
7. Rittenhouse, S. E., King, W. G., Downes, C. P. & Kucera, G. L. (1990) in Biology of Cellular Transducing Signals '89 (Vanderhoek, J. Y., ed.), pp. 227–233, Raven Press, New York
8. Nolan, R. D. & Lapetina, E. G. (1990) J. Biol. Chem. **265**, 2441–2445
9. Kaplan, D. R., Whitman, M., Schaffhausen, B., Pallas, D. C., White, M., Cantley, L. & Roberts, T. M. (1987) Cell **50**, 1021–1029
10. Whitman, M., Downes, C. P., Keeler, M., Keller, T. & Cantley, L. (1988) Nature (London) **332**, 644–646
11. Williams, L. T. (1989) Science **243**, 1564–1570
12. Whitman, M. & Cantley, L. (1988) Biochim. Biophys. Acta **948**, 327–344
13. Knight, D. K., Niggli, V. & Scrutton, M. C. (1984) Eur. J. Biochem. **143**, 437–446
14. Watson, S. P., McNally, J., Shipman, L. J. & Godfrey, P. P. (1988) Biochem. J. **249**, 345–350
15. Jakobs, K. H., Bauer, S. & Watanabe, Y. (1985) Eur. J. Biochem. **151**, 425–430
16. King, W. G. & Rittenhouse, S. E. (1989) J. Biol. Chem. **264**, 6070–6074
17. Kucera, G. L. & Rittenhouse, S. E. (1988) Biochem. Biophys. Res. Commun. **153**, 417–421
18. Fabiato, A. & Fabiato, F. (1979) J. Physiol. (Paris) **75**, 463–505
19. Carpenter, C., Duckworth, B. C., Auger, K. R., Cohen, B., Schaffhausen, B. S. & Cantley, L. C. (1990) J. Biol. Chem. **265**, 19704–19711
20. Banga, H. S., Walker, R. K., Winberry, L. K. & Rittenhouse, S. E. (1987) J. Biol. Chem. **262**, 14871–14874
21. Banga, H. S., Walker, R. K., Winberry, L. K. & Rittenhouse, S. E. (1988) Biochem. J. **252**, 297–300
22. Laemmli, U. K. (1970) Nature (London) **227**, 680–685
23. Lipsich, L. A., Lewis, A. J. & Brugge, J. S. (1983) J. Virol. **48**, 352–360
24. Golden, A., Nemeth, S. P. & Brugge, J. S. (1986) Proc. Natl. Acad. Sci. U.S.A. **83**, 852–856
25. Tsukuda, M., Asaoka, Y., Sekiguchi, K., Kikkawa, U. & Nishizuka, Y. (1988) Biochem. Biophys. Res. Commun. **155**, 1387–1395
26. House, C. & Kemp, B. E. (1987) Science **238**, 1726–1728
27. Kemp, B. E., Pearson, R. B., House, C., Robinson, P. J. & Means, A. R. (1989) Cell. Signalling **1**, 303–311
28. Huang, R.-S., Kucera, G. L. & Rittenhouse, S. E. (1991) J. Biol. Chem. **266**, 1652–1655
29. Rittenhouse, S. E. & Sasson, J. P. (1985) J. Biol. Chem. **260**, 8657–8660
30. Zavoico, G. B., Halenda, S. P., Sha'afi, R. I. & Feinstein, M. B. (1985) Proc. Natl. Acad. Sci. U.S.A. **82**, 3859–3862
31. Rittenhouse, S. E., Banga, H. S., Sasson, J. P., King, W. G. & Tarver, A. P. (1988) Philos. Trans. R. Soc. London **320**, 299–311

32. Cohen, P., Holmes, C. F. B. & Tsukitomi, Y. (1990) Trends Biochem. Sci. **15**, 98–102
33. Siffert, W., Siffert, G. & Scheid, P. (1987) Biochem. J. **241**, 301–303
34. Golden, A. & Brugge, J. S. (1989) Proc. Natl. Acad. Sci. U.S.A. **86**, 901–905
35. Brychaert, M. C., Rendu, R., Tobelem, G. & Wasteson, A. (1989) J. Biol. Chem. **264**, 4336–4341
36. Katada, T., Bokoch, G. M., Northup, J. K., Ui, M. & Gilman, A. G. (1984) J. Biol. Chem. **259**, 3568–3577
37. Banga, H. S., Simons, E. R., Brass, L. F. & Rittenhouse, S. E. (1986) Proc. Natl. Acad. Sci. U.S.A. **83**, 9197–9201
38. Carlson, K. E., Brass, L. F. & Manning, D. R. (1989) J. Biol. Chem. **264**, 13298–13305
39. Trahey, M. & McCormick, F. (1987) Science **238**, 542–544
40. Wolfman, A. & Macara, I. G. (1990) Science **248**, 67–69
41. Lips, D. L. & Majerus, P. W. (1989) J. Biol. Chem. **264**, 19911–19915
42. Yamamoto, K., Graziani, A., Carpenter, C., Cantley, L. C. & Lapetina, E. G. (1990) J. Biol. Chem. **265**, 22086–22089

Received 28 February 1991/18 April 1991; accepted 2 May 1991