Translocation-independent activation of protein kinase C by platelet-activating factor, thrombin and prostacyclin

Lack of correlation with polyphosphoinositide hydrolysis in rabbit platelets

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The relationship between polyphosphoinositide hydrolysis and protein kinase C (PKC) activation was explored in rabbit platelets treated with the agonists platelet-activating factor (PAF), thrombin and 12-*O*-tetradecanoylphorbol 13-acetate (TPA), and with the anti-aggregant prostacyclin (PGI₂). Measurement of the hydrolysis of radiolabelled inositol-containing phospholipids relied upon the separation of the products [³H]inositol mono-, bis- and tris-phosphates by Dowex-1 chromatography. PKC activity, measured in platelet cytosolic and Nonidet-P40-solubilized particulate extracts that were fractionated by MonoQ chromatography, was based upon the ability of the enzyme to phosphorylate either histone H1 in the presence of the activators Ca²⁺, diacylglycerol and phosphatidylserine, or protamine in the absence of Ca²⁺ and lipid. Treatment of platelets for 1 min with PAF (2 nM) or thrombin (2 units/ml) led to the rapid hydrolysis of inositol-containing phospholipids, a 2–3-fold stimulation of both cytosolic and particulate-derived PKC activity, and platelet aggregation. Exposure to TPA (200 nM) for 5 min did not stimulate formation of phosphoinositides, but translocated more than 95% of cytosolic PKC into the particulate fraction, and induced a slower rate of aggregation. PGI₂ (1 µg/ml) did not enhance phosphoinositide production, and at higher concentrations (50 µg/ml) it antagonized the ability of PAF, but not that of thrombin, to induce inositol phospholipid turnover, even though platelet aggregation in response to both agonists was blocked by PGI₂. On the other hand, PGI₂ alone also appeared to activate (by 3–5-fold) cytosolic and particulate PKC by a translocation-independent mechanism. The activation of PKC by PGI₂ was probably mediated via cyclic AMP (cAMP), as this effect was mimicked by the cAMP analogue 8-chlorophenylthio-cAMP. It is concluded that this novel mechanism of PKC regulation by platelet agonists may operate independently of polyphosphoinositide turnover, and that activation of cAMP-dependent protein kinase represents another route leading to PKC activation.

INTRODUCTION

Nearly a decade ago, Yasutomi Nishizuka and his colleagues proposed that thrombin-triggered diacylglycerol (DAG) production from phosphatidylinositol (PI) in human platelets was linked to the activation of the Ca²⁺-activated phosphatidylserine (PS)-dependent protein kinase (PKC) (Kawahara *et al.*, 1980). Subsequently PKC was implicated in the signal transduction pathways of a wide spectrum of cytokines that also evoke PI turnover (Nishizuka, 1986, 1988). This model was further consolidated with the discovery that the extremely rapid agonist-induced hydrolysis of phosphatidylinositol 4,5-bisphosphate (PIP₂) by phospholipase C also generated inositol trisphosphate (IP₃), an intracellular mobilizer of Ca²⁺ from the endoplasmic reticulum, which could act synergistically with DAG to activate PKC (Berridge, 1987). Based upon a plethora of studies in the literature it is widely held that DAG, and its tumour-promoting phorbol ester mimetic 12-*O*-tetradecanoylphorbol 13-acetate (TPA), facilitate the conversion of inactive cytosolic PKC into a membrane-bound active form.

The pivotal role that PKC plays in platelet activation by various agonists is implied by the ability of a range of PKC inhibitors to block platelet responses following challenge with

thrombin, platelet-activating factor (PAF) or phorbol ester (Hannun *et al.*, 1987; Yamada *et al.*, 1987; Schachtele *et al.*, 1988; Watson *et al.*, 1988; Watson & Hambleton, 1989), although the use of sphingosine has been criticized in this context (Krishnamurthi *et al.*, 1989). Thrombin and PAF are the most potent platelet activators known, and they can induce aggregation within 1 min (Huang & Detwiler, 1986). By contrast, TPA-induced platelet aggregation is relatively slow, but it may act synergistically with sub-threshold levels of thrombin to trigger rapid activation (Yatomi *et al.*, 1987; Watson & Hambleton, 1989). In rabbit platelets, thrombin and PAF both elicit the production of DAG and IP₃ from PIP₂ (Shukla *et al.*, 1987). Apart from mobilizing Ca²⁺ from intracellular stores via IP₃, thrombin also stimulates the influx of extracellular Ca²⁺ (Jy & Haynes, 1987). Based upon enhanced binding of radiolabelled phorbol ester to thrombin-treated (Crouch & Lapetina, 1988) and PAF-treated (Siess & Lapetina, 1988) human platelets, the rise in cytoplasmic Ca²⁺ has been proposed to facilitate translocation of PKC to the plasma membrane of these cells. O'Flaherty & Nishihira (1987) were able to observe a decrease in soluble phorbol-ester binding and PKC activity in PAF-treated human neutrophils, provided that these cells were also exposed to cytochalasin B. Furthermore, PAF exhibited a synergistic

Abbreviations used: cAMP, cyclic AMP (adenosine 3',5' monophosphate); CPT-cAMP, 8-chlorophenylthio-cAMP; DAG, diacylglycerol; G-protein; GTP-binding protein; IP, inositol monophosphate; IP₂, inositol bisphosphate; IP₃, inositol trisphosphate; NP40, Nonidet P-40; p47, 40–47 kDa protein; PAF, platelet-activating factor; PGI₂, prostacyclin (prostaglandin I₂); PI, phosphatidylinositol; PIP₂, phosphatidylinositol 4,5-bisphosphate; PKA, cAMP-dependent protein kinase; PKC, protein kinase C; PKIP, cAMP-dependent protein kinase inhibitor peptide; PS, phosphatidylserine; TPA, 12-*O*-tetradecanoylphorbol 13-acetate.

effect with sub-threshold levels of TPA to deplete cytosolic PKC in these cells (O'Flaherty *et al.*, 1989).

Prostacyclin (PGI₂) is a powerful inhibitor of platelet aggregation in response to thrombin and other agonists (Moncada *et al.*, 1976). Via activation of adenylate cyclase, it elevates the level of cyclic AMP (cAMP) in platelets, and this leads to a stimulation of cAMP-dependent protein kinase (PKA) activity. At least two sites of PKA action in the disruption of agonist-induced platelet aggregation have been distinguished, and these are pre- and post-PKC activation (Kroll *et al.*, 1988; Siess & Lapetina, 1989). PGI₂ and cAMP-analogue treatment of platelets leads to an inhibition of agonist-stimulated PIP₂ breakdown, perhaps via PKA phosphorylation of a putative GTP-binding protein (G-protein) which couples the agonist receptors to phospholipase C (Halenda & Feinstein, 1984; Ieyasu *et al.*, 1982; Doni *et al.*, 1988; Lazarowski & Lapetina, 1989; Yada *et al.*, 1989). Likewise, activators of PKC such as TPA and synthetic DAGs also inhibit agonist-induced PIP₂ hydrolysis in human platelets by an undefined mechanism (Watson & Lapetina, 1985; Tohmatsu *et al.*, 1986; Doni *et al.*, 1988; Baldassare *et al.*, 1989).

In a recent study (Pelech *et al.*, 1990), we were unable to demonstrate PKC translocation in PAF-treated rabbit platelets as assessed by binding of radiolabelled phorbol ester. However, within 1 min of PAF addition to the cells, a 2–3-fold increase in both cytosolic and particulate PKC activity was detectable. The mechanism leading to the stimulation of total cellular PKC activity was not established, but the data were consistent with a possible covalent modification of the enzyme within or near the

catalytic domain (Pelech *et al.*, 1990). In the present study, we show that thrombin mimics the action of PAF in stimulating total platelet PKC activity. In addition, we have used PGI₂ to evaluate the requirement for polyphosphoinositide turnover in the thrombin- and PAF-induced activation of PKC.

EXPERIMENTAL

Materials

Prostacyclin (PGI₂) sodium salt, 1-*O*-hexadecyl-2-acetyl-*sn*-glycero-3-phosphocholine (PAF) (L-isomer), TPA, bovine thrombin (50–100 units/mg of protein), histone H1 (type III-S), protamine sulphate, PS, diolein, ATP, Nonidet P-40 (NP40), β -glycerophosphate, Mops, cAMP-dependent protein kinase inhibitor peptide (amino acids 5–24; TTYADFIASGRTGRRN-AIHD) (PKIP) (Cheng *et al.*, 1986; Scott *et al.*, 1986), and LiCl were bought from Sigma Chemical Co. (St. Louis, MO, U.S.A.). *myo*-[2-³H]inositol (10–20 Ci/mmol) was purchased from Amersham (Arlington Heights, IL, U.S.A.) and [γ -³²P]ATP was from ICN Radiochemicals.

Preparation of platelet extracts and MonoQ chromatography

Rabbit platelets were isolated according to the method of Pinckard *et al.* (1984). Platelets at a concentration of 1×10^8 /ml were challenged with PAF (2 nM), thrombin (2 units/ml), PGI₂ (1 or 50 μ g/ml) or TPA (200 nM) at 37 °C in Tyrode's buffer, pH 7.2. For the PKC assays, the platelets were sonicated following drug treatment (10–15 s at the 40 setting of a Sonics

Fig. 1. MonoQ f.p.l.c. of PKC from platelets treated with thrombin

Cells were untreated (○) or exposed to 2 units of thrombin/ml (□) for 1 min prior to harvesting. MonoQ chromatography of 0.5 mg of cytosolic protein (a and c) and 0.5 mg of NP40-solubilized particulate protein (b and d) from platelet extracts was performed, and the column fractions were assayed for phosphorylating activity as described in the Experimental section. Panels (a) and (b) show the histone H1-phosphorylating activity in the presence of Ca²⁺, PS and diolein. Histone H1-phosphorylating activity in the column fractions never exceeded 300 pmol/min per ml in the absence of Ca²⁺, PS and diolein. Panels (c) and (d) show the protamine-phosphorylating activities in the column fractions measured in the absence of Ca²⁺ and added lipid. Between the untreated and treated extracts, the recoveries of applied phosphorylating activities illustrated in each panel were very comparable (approx. 60–80%). Similar results were obtained in at least three independent experiments.

Vibra-cell) in 0.5 ml of buffer A (75 mM- β -glycerophosphate/20 mM-Mops (pH 7.2)/15 mM-EGTA/2 mM-EDTA/1 mM-sodium orthovanadate/1 mM-dithiothreitol). The homogenate was subjected to 200000 *g* for 15 min in a Beckman TL-100 ultracentrifuge, and the supernatant (cytosol) (~4 mg of protein/ml) was immediately divided into portions and frozen at -70°C . The pellet was again sonicated in 0.5 ml of buffer A containing 1% NP40, and the detergent-solubilized extract (~1 mg of protein/ml) was obtained following centrifugation at 200000 *g* for 15 min and also stored at -70°C .

For anion-exchange chromatography, 500 μg of extract protein was loaded on to a 1 ml MonoQ (Pharmacia) column and eluted at a flow rate of 0.8 ml/min into 0.25 ml fractions with a 10 ml linear gradient of 0–0.8 M-NaCl in buffer B (25 mM- β -glycerophosphate/10 mM-Mops (pH 7.2)/5 mM-EGTA/2 mM-EDTA/1 mM sodium orthovanadate/1 mM-dithiothreitol).

Kinase and protein assays

The thawed extracts and column fractions were assayed for phosphorylating activity with the following components for 5 min at 30°C in a volume of 25 μl : histone H1 (Sigma type IIS) or protamine sulphate (1 mg/ml), 50 μM -[γ - ^{32}P]ATP (1500 c.p.m./pmol), 25 mM- β -glycerophosphate, 10 mM-Mops, pH 7.2, 15 mM-MgCl₂, 2 mM-EGTA, 2 mM-EDTA, 1 mM-sodium orthovanadate, 1 mM-dithiothreitol and 500 nM-PKIP. Where stated, the incubations also included 4.5 mM-CaCl₂ (~0.8 mM free Ca²⁺) 60 mg of PS/ml and 6 mg of diolein/ml. At the conclusion of the reaction period, 20 μl portions were spotted on to 2 cm² pieces of Whatman P81 phosphocellulose paper and washed 30 s later with several changes of 1% (v/v) phosphoric acid. The wet filter papers were transferred into 6 ml plastic scintillation vials that contained 2 ml of Ecolume (ICN) scintillation fluid and were analysed for radioactivity in a Packard liquid scintillation counter. Protein was estimated by the method of Bradford (1976) with BSA ($A_{280}^{1\%}$ 6.5) as the standard.

Polyphosphoinositide turnover studies

Rabbit platelets ($3 \times 10^9/\text{ml}$) were incubated with 100 μCi of *myo*-[2- ^3H]inositol/ml in Tyrode's buffer, pH 7.0, in the presence of 1 μg of PGI₂/ml with 0.1 mM-EGTA and without Ca²⁺ for a period of 3 h at 37°C . Subsequently, cells were washed twice to remove unincorporated radiolabelled inositol, and the cells were diluted in Tyrode's pH 7.2 buffer containing 12 mM-LiF, and 1.3 mM-CaCl₂ in the absence of EGTA. Cells (0.5 ml) were challenged with drugs for 1 min, and then 1.8 ml of denaturing buffer was added as described by Watson *et al.* (1984).

The inositol phosphates were extracted by adding 0.6 ml of water to the samples to separate the phases. Of the upper phase 1.5 ml was further diluted with 2.5 ml of water and loaded on to a 1 ml Dowex-1 (Bio-Rad) column (pre-equilibrated in water). The inositol phosphates were separated when the Dowex column was washed sequentially with the following solutions (Watson *et al.*, 1985). Free inositol was eluted when the column was washed with 16 ml of 60 mM-ammonium formate/5 mM-disodium tetraborate; inositol monophosphate (IP) was released with 16 ml of 200 mM-ammonium formate/100 mM-formic acid; inositol bisphosphate (IP₂) was eluted with 20 ml of 400 mM-ammonium formate/100 mM-formic acid; and inositol trisphosphate (IP₃) was recovered with 12 ml of 1 M-ammonium formate/100 mM-formic acid. The radioactivity was quantified in 8 ml portions of the column eluates.

Platelet aggregation assay

Washed rabbit platelets ($2 \times 10^8/\text{ml}$) were prepared in Tyrode's buffer, pH 7.2, containing CaCl₂ (1.3 mM). Aliquots (0.5 ml) were assayed for drug-induced aggregation at 37°C as described

Fig. 2. Effect of TPA on agonist-induced polyphosphoinositide turnover

Rabbit platelets that were prelabelled with *myo*-[2- ^3H]inositol were subsequently incubated in the absence (■) or presence of 200 nM-TPA for 1 min (▨) or 30 min (▩), and then harvested immediately (control) or treated for an additional 1 min with 2 nM-PAF or 2 units of thrombin/ml prior to harvesting as detailed in the Experimental section. The inositol phosphates were extracted from the platelets and resolved by Dowex-1 column chromatography. The incorporation of radioactivity into the various polyphosphoinositide metabolites was expressed as a percentage of the total radioactivity recovered in free inositol, IP, IP₂ and IP₃ together. Values are the means $\pm$ S.D. of three to eight independent determinations.

by Pinckard *et al.* (1984) using a Bio-Data aggregometer (Bio-Data, Hatboro, PA, U.S.A.). Drugs (PAF, thrombin, PGI₂ or TPA) in 50 μl of 0.25% BSA in Tyrode's buffer were added directly to the platelets in a cuvette. Activities were measured as percentage increase in light transmission.

Serotonin release assay

Washed rabbit platelets ($2 \times 10^8/\text{ml}$) were incubated with [^3H]5-hydroxytryptamine (serotonin) (0.3 $\mu\text{Ci}/\text{ml}$) for 3 h at 37°C . The non-incorporated [^3H]serotonin was removed by washing the platelets three times with Tyrode's buffer. The platelets ($2 \times 10^8/\text{ml}$) were challenged with various agents for 1 min (PAF, thrombin, PGI₂) or 30 min (TPA), and subsequently

Fig. 3. Effect of TPA on PKC activation by PAF and thrombin

Washed platelets were harvested either immediately (■) or after incubation with 200 nM-TPA for 15 min and then in the absence (▨) or presence of 2 nM-PAF (▤) or 2 units of thrombin/ml (▥) for a further 1 min. MonoQ chromatography of 0.5 mg of cytosolic protein (a and b) or 0.5 mg of NP40-solubilized particulate protein (c and d) from platelet extracts was performed, and the column fractions were assayed for phosphorylating activity as described in the Experimental section. Panels (a) and (c) show the peak histone H1-phosphorylating activity in the presence of Ca^{2+} , PS and diolein in the pooled column fractions (0.26–0.34 M-NaCl). Panels (b) and (d) show the protamine-phosphorylating activities measured in the absence of Ca^{2+} and added lipid in the same pooled peak column fractions. Values are expressed relative to the control PKC activity (100 %) in each panel, and are the mean ($\pm$ S.D. where appropriate) of two to four independent experiments.

pelleted in a microcentrifuge at 15000 rev./min for 15 s. The radioactivity remaining in the supernatant was quantified and plotted against the initial radioactivity present in the cells.

RESULTS

PAF- and thrombin-induced PIP_2 hydrolysis and PKC activation

Rabbit platelets are particularly sensitive to activation by picomolar concentrations of PAF. Within 30 s, a saturating dose of PAF (i.e. 2 nM) can elicit aggregation and the release of approx. 80 % of total cellular serotonin (results not shown). In a previous study (Pelech *et al.*, 1990), we found that when precautions were adopted to facilitate rapid breakage and subcellular fractionation of PAF-treated platelets (see the Experimental section), this agonist also induced a 2–3-fold increase in total cellular PKC activity. These changes were most evident after anion-exchange chromatography of the cytosolic and NP40-solubilized particulate extracts of the cells. As PAF and thrombin appear to share similar signal transduction pathways in platelets, we were interested by the prospect that thrombin might mimic this action of PAF on PKC activity levels. As illustrated in Fig. 1, prior treatment of platelets with 2 units of thrombin/ml resulted in an almost 4-fold increase in both cytosolic and particulate-derived PKC activity (eluting at ~ 0.3 M-NaCl from MonoQ) as compared with non-treated cells. This enhancement in PKC activity caused by thrombin was evident regardless of whether PKC was assayed by its ability to phosphorylate either histone H1 in the presence of Ca^{2+} , DAG and PS or protamine sulphate in the absence of these components (Bazzi & Nelsestuen, 1987). In general, thrombin-induced elevation of PKC activity was slightly more pronounced than the PAF-induced changes.

As described previously for human (Watson & Lapetina, 1985) and rabbit (Shukla *et al.*, 1987) platelets, thrombin similarly evoked a larger stimulation of inositol-containing-phospholipid hydrolysis than did PAF (Fig. 2). Thrombin produced 4–10-fold

increases in radiolabelling of IP , IP_2 and IP_3 , whereas PAF elicited only 2–4-fold more incorporation of ^3H label into the various inositol phosphate species.

While the second messengers of PIP_2 hydrolysis have been linked with PKC activation, studies with human platelets have also pointed towards a feedback inhibitory role for PKC in the regulation of PIP_2 -specific phospholipase C (Watson & Lapetina, 1985; Tohmatsu *et al.*, 1986). Preincubation of platelets for 1 min with TPA had no significant effect on the ability of thrombin or PAF to induce PIP_2 degradation (Fig. 2). However, extended exposure of the cells to the phorbol ester for 30 min did attenuate both IP_3 formation in response to PAF and thrombin-induced IP_2 and IP_3 production. Prolonged treatment of platelets with TPA also induced a slow rate of aggregation that was maximal after 30 min, at which time about 50–70 % of the cellular serotonin was released (results not shown).

We have found that treatment of rabbit platelets with TPA for only 1 min is sufficient for essentially complete translocation of PKC from the cytosolic to the particulate fraction of cells, and presumably for activation of the kinase (results not shown). Such a quantitative redistribution of PKC in 15-min-TPA-treated platelets is shown in Fig. 3, where the cytosolic PKC activity disappeared and the particulate-derived PKC activity more than doubled. Since thrombin and PAF apparently stimulated PKC activity via a translocation-independent mechanism, it was intriguing to evaluate the combined effects of these agonists with TPA on the PKC activity and distribution. In cells that were pretreated for 15 min with 200 nM-TPA and then exposed for an additional 1 min to either 2 units of thrombin/ml or 2 nM-PAF, there were no additional increases in the particulate PKC activities towards histone H1 or protamine. On the other hand, there were several-fold increases in the residual cytosolic PKC activity after addition of thrombin or PAF to the cells preincubated with TPA (Fig. 3). A possible interpretation of these findings is that TPA alone induces an increase in total PKC activity, like PAF and thrombin, but unlike these agents, the

Fig. 4. Effect of PGI₂ on agonist-induced polyphosphoinositide turnover

Rabbit platelets that were prelabelled with *myo*-[2-³H]inositol were incubated subsequently in the absence (■) or presence of PGI₂ [1 µg/ml (▨) or 50 µg/ml (▩)] for 1 min, and then harvested immediately (control) or treated for an additional 1 min with 2 nM-PAF or 2 units of thrombin/ml prior to harvesting as detailed in the Experimental section. The inositol phosphates were extracted from the platelets and resolved by Dowex-1 column chromatography. The incorporation of radioactivity into the various polyphosphoinositide metabolites was expressed as a percentage of the total radioactivity recovered in free inositol, IP, IP₂ and IP₃ together. Values are the means ± S.D. of three to eight independent determinations.

phorbol ester also induces translocation of PKC. As the membrane-associated form of PKC is especially sensitive to proteolytic degradation (Nishizuka, 1986), a net increase in total PKC activity in phorbol-ester-treated cells may not be generally evident.

Action of PGI₂ on PAF- and thrombin-induced inositol-containing-phospholipid hydrolysis and platelet aggregation

PGI₂ is among the most powerful anti-aggregants known (Moncada *et al.*, 1976). Preincubation of rabbit platelets for 1 min with 1 µg of PGI₂/ml was sufficient to block thrombin-

induced, but not PAF-induced, aggregation (results not shown). However, at 50 µg/ml, PGI₂ also prevented platelet aggregation in response to PAF. Surprisingly, thrombin-induced inositol-containing-phospholipid hydrolysis was relatively insensitive to inhibition by up to 50 µg of PGI₂/ml, whereas PAF-initiated breakdown was severely attenuated with 1 µg of PGI₂/ml (Fig. 4).

Post-receptor signal transduction by PGI₂ is mediated via cAMP production and activation of PKA. The addition of a cAMP analogue, 8-chlorophenylthio-cAMP (CPT-cAMP) at 0.5 mM for 5 min to rabbit platelets had no effect on basal inositol-containing-phospholipid hydrolysis; similarly to PGI₂, it decreased PAF-induced production of polyphosphoinositides (results not shown). Recent reports have implicated cAMP-mediated PKA phosphorylation of a G-protein that modulates phospholipase C activity as the site of inhibition of agonist-induced PIP₂ hydrolysis (Doni *et al.*, 1988; Yada *et al.*, 1989; Lazarowski & Lapetina, 1989).

Action of PGI₂ on PKC activation

In light of the thrombin- and PAF-induced increases in PKC activity as measured in Fig. 1 of this study, it was interesting to test whether PGI₂ could inhibit these responses. Surprisingly, as shown in Fig. 5, incubation of rabbit platelets for 1 min with 50 µg of PGI₂/ml alone evoked the same increases in cytosolic and particulate-derived PKC activities towards histone H1 and protamine as noted for PAF and thrombin. Treatment of platelets with the combinations of PGI₂ with either PAF or thrombin produced no further stimulation of the cytosolic and particulate-derived PKC activities than was seen with these agents separately (Fig. 6). This indicated that, despite the differences in second messenger production in response to PGI₂ and the agonists PAF and thrombin, these agents induced translocation-independent activation of PKC via a convergent mechanism. That the ability of PGI₂ to activate PKC was indeed mediated through cAMP was confirmed in experiments with the cAMP analogue CPT-cAMP (Fig. 7). Following incubation of platelets with 0.5 mM-CPT-cAMP for 10 min, and MonoQ chromatography of the cytosolic and NP40-solubilized particulate extracts of these cells, PKC activity in these fractions was found to be increased by 3–7-fold (Fig. 7).

DISCUSSION

In the present study with rabbit platelets, we have investigated the roles of PIP₂ turnover and PKC activation in the signal transduction pathways of thrombin and PAF. Rabbit platelets are much more sensitive to PAF than are human platelets. However, much of our data with regard to the stimulatory effects of thrombin and PAF and the inhibitory actions of TPA and PGI₂ on inositol-containing-phospholipid breakdown were in accordance with similar studies performed with human platelets. Rabbit platelets were also a simpler model system for the study of PKC, as they appear to contain mostly the β -isoenzyme (Pelech *et al.*, 1990). Human platelets, by contrast, also have high levels of the α -isoenzyme of PKC (Tsukuda *et al.*, 1988; Watanabe *et al.*, 1988). Although PKC has long been implicated in the actions of thrombin and PAF, most of the evidence for their activation of PKC has been indirect, relying largely on the suppressive effects of PKC inhibitors.

Numerous studies have documented the phosphorylation of a 40–47 kDa protein (p47) in platelets in response to thrombin, PAF and phorbol esters (Kawahara *et al.*, 1980; Ieyasu *et al.*, 1982; Sano *et al.*, 1983). Furthermore, experiments with a variety of PKC inhibitors indicate that p47 is a specific substrate for PKC (Yamada *et al.*, 1987; Watson *et al.*, 1988; Krishnamurthi

Fig. 5. MonoQ f.p.l.c. of PKC from platelets treated with PGI_2

Cells were untreated (Δ , $\circ$) or exposed to $50 \mu\text{g}$ of PGI_2/ml ($\blacktriangle$, $\bullet$) for 1 min prior to harvesting. MonoQ chromatography of 0.5 mg of cytosolic protein (a and c) and 0.5 mg of NP40-solubilized particulate protein (b and d) from platelet extracts was performed, and the column fractions were assayed for phosphorylating activity as described in the Experimental section. Panels (a) and (b) show the histone H1-phosphorylating activity in the absence ($\circ$, $\bullet$) and presence (Δ , $\blacktriangle$) of Ca^{2+} , PS and diolein. Panels (c) and (d) show the protamine-phosphorylating activities in the column fractions measured in the absence of Ca^{2+} and added lipid. Similar results were obtained in three independent experiments.

et al., 1989). The observation that phosphorylation of p47 is increased in thrombin- and PAF-treated platelets has been used to argue that agonist-induced PIP_2 turnover is coupled to PKC activation (Ieyasu *et al.*, 1982; Sano *et al.*, 1983). However, PGI_2 and cAMP-analogue treatments of platelets block PAF-, thrombin- and fluoride-induced p47 phosphorylation (Ieyasu

et al., 1982; Sano *et al.*, 1983; Kroll *et al.*, 1988; Siess & Lapetina, 1989; Lazarowski & Lapetina, 1989), even though PIP_2 breakdown in response to thrombin was not sensitive to up to $50 \mu\text{g}$ of PGI_2/ml (Fig. 4). Therefore p47 phosphorylation may not serve as an accurate measure of PKC activity in platelets.

In the present study, we have found that PGI_2 and cAMP-

Fig. 6. Effect of PGI_2 on PKC activation by PAF and thrombin

Washed platelets were either harvested immediately (control) or after incubation with $50 \mu\text{g}$ of PGI_2/ml for 1 min or in some cases after an additional 1 min in the absence or presence of 2 nM-PAF or 2 units of thrombin/ml. MonoQ chromatography of 0.5 mg of cytosolic protein (a and b) and 0.5 mg of NP40-solubilized particulate protein (c and d) from platelet extracts was performed, and the column fractions were assayed for phosphorylating activity as described in the Experimental section. Panels (a) and (c) show the peak histone H1-phosphorylating activity in the presence of Ca^{2+} , PS and diolein in the pooled peak column fractions (0.26–0.34 M-NaCl). Panels (b) and (d) show the protamine-phosphorylating activities measured in the absence of Ca^{2+} and added lipid in the same pooled peak column fractions. Values are expressed relative to the control PKC activity (100%) in each panel, and are the mean ($\pm$ S.D. where appropriate) of two to seven independent experiments. $\square$, Control; $\square$ (hatched), + PAF; $\blacksquare$, + thrombin; $\square$ (diagonal lines), + PGI_2 ; $\square$ (cross-hatched), + PAF + PGI_2 ; $\blacksquare$, + thrombin + PGI_2 .

Fig. 7. MonoQ f.p.l.c. of PKC from platelets treated with a cAMP analogue

Cells were untreated (Δ , $\circ$) or exposed to 0.5 mM-CPT-cAMP ($\blacktriangle$, $\bullet$) for 5 min prior to harvesting. MonoQ chromatography of 0.5 mg of cytosolic protein (a and c) or 0.5 mg of NP40-solubilized particulate protein (b and d) from platelet extracts were performed, and the column fractions were assayed for phosphorylating activity as described in the Experimental section. Panels (a) and (b) show the histone H1-phosphorylating activity in the absence ($\circ$, $\bullet$) and presence (Δ , $\blacktriangle$) of Ca^{2+} , PS and diolein. Panels (c) and (d) show the protamine-phosphorylating activities in the column fractions measured in the absence of Ca^{2+} and added lipid. Similar results were obtained in three independent experiments.

analogue treatments of rabbit platelets, like PAF and thrombin, result in 2-fold or greater increases in total cellular PKC activity. This is not easily reconciled with the inhibitory effects of these anti-aggregants on PAF- and thrombin-induced p47 phosphorylation. A possible explanation for this conundrum is that PKA phosphorylation of p47 increases its apparent size. Phosphorylation-induced retardation of proteins on SDS/PAGE gels has been described previously (Casnellie, 1987). PGI_2 treatment leads to the appearance of a 50 kDa phosphoprotein on SDS/PAGE gels of platelet extracts (Siess & Lapetina, 1989). Analysis of the amino acid sequence of the p47 protein reveals the presence of at least five classic consensus sequences for PKA phosphorylation (i.e. two basic residues on the *N*-terminal side of a phosphorylatable serine or threonine residue) (Tyers *et al.*, 1988). A shift in the apparent size of p47 to approx. 50 kDa could account for how cAMP analogues seem to block even TPA-induced phosphorylation of p47 (Kroll *et al.*, 1988), as PKA has not been shown to directly inhibit PKC activity. Our findings imply that PKA may actually stimulate PKC activity in platelets, but further experiments are necessary to determine whether this is a direct effect.

In a recent study (Pelech *et al.*, 1990), we discovered that treatment of rabbit platelets with PAF for 1 min led to 2–3-fold increases in both soluble and particulate-derived PKC activities, with no apparent changes in the levels of phorbol ester binding. We have now shown that such a translocation-independent mechanism of PKC activation is also utilized by thrombin (Fig. 1) and apparently by PGI_2 (Fig. 5). These results appear to

conflict with the findings of Siess & Lapetina (1988), who described 2-fold increases in phorbol ester binding to intact human platelets within 10 s of treatment with PAF. However, it may be that only the plasma-membrane-bound contingent of the particulate-associated PKC was examined in this latter study. The occurrence of a corresponding decrease in soluble phorbol ester binding activity in PAF-treated human platelets was not evaluated (Siess & Lapetina, 1988).

The molecular basis for PAF-, thrombin- and PGI_2 -induced PKC activation remains obscure. However, the size of PKC by gel filtration on Superose 6/12 (approx. 80 kDa) was not altered following PAF treatment of rabbit platelets (Pelech *et al.*, 1990). Furthermore, the stimulation of PKC activity was stable to detergent extraction and anion-exchange chromatography. It is tempting to speculate that the effects of PAF, thrombin and PGI_2 on PKC might be mediated via a covalent modification, such as phosphorylation of the enzyme. Recent studies (Golden & Brugge, 1989; Nakamura & Yamamura, 1989) have demonstrated that thrombin can stimulate protein-tyrosine phosphorylation in human platelets. We have also detected increased protein-tyrosine phosphorylation in rabbit platelets in response to PAF (H. Salari, V. Duronio & S. L. Pelech, unpublished work). It will be of interest to test the ability of protein-tyrosine kinase inhibitors (Yaish *et al.*, 1988) to block the PAF-, thrombin- and PGI_2 -induced activations of PKC.

The ability of PGI_2 to block thrombin-induced aggregation in rabbit platelets, whilst having little effect on inositol-containing-phospholipid hydrolysis, implied a site of action of PGI_2 down-

stream of this event. Indeed, recent studies with human platelets (Doni *et al.*, 1988; Kroll *et al.*, 1988; Siess & Lapetina, 1989) have indicated that PGI₂ can also function at steps distal to PKC activation. On the other hand, PAF signal transduction leading to aggregation can apparently operate independently of polyphosphoinositide turnover (Fig. 4).

S. P. was the recipient of a Medical Research Council of Canada Scholarship. This research was supported by grants from The British Columbia Heart Foundation and The British Columbia Health Care Research Foundation.

REFERENCES

- Baldassare, J. J., Henderson, P. A. & Fisher, G. J. (1989) *Biochemistry* **28**, 56–60.
- Bazzi, M. D. & Nelsestuen, G. L. (1987) *Biochem. Biophys. Res. Commun.* **147**, 248–253.
- Berridge, M. J. (1987) *Biochim. Biophys. Acta* **907**, 33–45.
- Bradford, M. M. (1976) *Anal. Biochem.* **72**, 248–254.
- Casnellie, J. E. (1987) *J. Biol. Chem.* **262**, 9859–9864.
- Cheng, H. C., Kemp, B. E., Pearson, R. B., Smith, A. J., Misconi, L., Van Patten, S. M. & Walsh, D. A. (1986) *J. Biol. Chem.* **261**, 989–992.
- Crouch, M. F. & Lapetina, E. G. (1988) *Biochem. Biophys. Res. Commun.* **156**, 149–156.
- Doni, M. G., Deana, R., Bertocello, S., Zoccarato, F. & Alexandre, A. (1988) *Biochem. Biophys. Res. Commun.* **156**, 1316–1323.
- Golden, A. & Brugge, J. S. (1989) *Proc. Natl. Acad. Sci. U.S.A.* **86**, 901–905.
- Halenda, S. P. & Feinstein, M. B. (1984) *Biochem. Biophys. Res. Commun.* **124**, 507–513.
- Hannun, Y. A., Greenberg, C. S. & Bell, R. M. (1987) *J. Biol. Chem.* **262**, 13620–13626.
- Huang, E. M. & Detwiler, T. C. (1986) in *Biochemistry of Platelets* (Phillips, D. R. & Shuman, M. A., eds.), pp. 1–68, Academic Press, New York.
- Ieyasu, H., Takai, Y., Kaibuchi, K., Sawamura, M. & Nishizuka, Y. (1982) *Biochem. Biophys. Res. Commun.* **108**, 1701–1708.
- Jy, W. & Haynes, D. H. (1987) *Biochim. Biophys. Acta* **929**, 88–102.
- Kawahara, Y., Takai, Y., Minakuchi, R., Sano, K. & Nishizuka, Y. (1980) *Biochem. Biophys. Res. Commun.* **97**, 309–317.
- Krishnamurthi, S., Patel, Y. & Kakkar, V. V. (1989) *Biochim. Biophys. Acta* **1010**, 258–264.
- Kroll, M. H., Zavoico, G. B. & Schafer, A. I. (1988) *Biochim. Biophys. Acta* **970**, 61–67.
- Lazarowski, E. R. & Lapetina, E. G. (1989) *Biochem. Biophys. Res. Commun.* **158**, 440–444.
- Moncada, S., Gryglewski, R., Bunting, S. & Vane, J. R. (1976) *Nature* (London) **263**, 663–665.
- Nakamura, S. & Yamamura, H. (1989) *J. Biol. Chem.* **264**, 7089–7091.
- Nishizuka, Y. (1986) *Science* **233**, 305–311.
- Nishizuka, Y. (1988) *Nature* (London) **334**, 661–665.
- O'Flaherty, J. T. & Nishihira, J. (1987) *J. Immunol.* **138**, 1889–1895.
- O'Flaherty, J. T., Jacobson, D. P. & Redman, J. F. (1989) *J. Biol. Chem.* **264**, 6836–6843.
- Pelech, S. L., Charest, D. L., Howard, S. L., Paddon, H. B. & Salari, H. (1990) *Biochim. Biophys. Acta* **1051**, 100–107.
- Pinckard, R. N., Farr, R. S. & Hanahan, D. G. (1984) *J. Immunol.* **123**, 1847–1853.
- Sano, K., Takai, Y., Yamanishi, J. & Nishizuka, Y. (1983) *J. Biol. Chem.* **258**, 2010–2013.
- Schachtele, C., Seifert, R. & Osswald, H. (1988) *Biochem. Biophys. Res. Commun.* **151**, 542–547.
- Scott, J. D., Glaccum, M. B., Fischer, E. H. & Krebs, E. G. (1986) *Proc. Natl. Acad. Sci. U.S.A.* **83**, 1613–1616.
- Shukla, S. D., Franklin, C. C. & Carter, M. G. (1987) *Biochim. Biophys. Acta* **929**, 134–141.
- Siess, W. & Lapetina, E. G. (1988) *Biochem. J.* **255**, 309–318.
- Siess, W. & Lapetina, E. G. (1989) *Biochem. J.* **258**, 57–65.
- Tohmatsu, T., Hattori, H., Nagao, S., Ohki, K. & Nozawa, Y. (1986) *Biochem. Biophys. Res. Commun.* **134**, 868–875.
- Tsukuda, M., Asaoka, Y., Sekiguchi, K., Kikkawa, U. & Nishizuka, Y. (1988) *Biochem. Biophys. Res. Commun.* **155**, 1387–1395.
- Tyers, M., Rachubinski, R. A., Stewart, M. I., Varrichio, A. M., Shorr, R. G. L., Haslam, R. J. & Harley, C. B. (1988) *Nature* (London) **333**, 470–473.
- Watanabe, M., Hagiwara, M., Onoda, K. & Hidaka, H. (1988) *Biochem. Biophys. Res. Commun.* **152**, 642–648.
- Watson, S. P. & Hambleton, S. (1989) *Biochem. J.* **258**, 479–485.
- Watson, S. P. & Lapetina, E. G. (1985) *Proc. Natl. Acad. Sci. U.S.A.* **82**, 2623–2626.
- Watson, S. P., McConnell, R. T. & Lapetina, E. G. (1984) *J. Biol. Chem.* **259**, 13199–13203.
- Watson, S. P., Reep, B., McConnell, R. T. & Lapetina, E. G. (1985) *Biochem. J.* **226**, 831–837.
- Watson, S. P., McNally, J., Shipman, L. J. & Godfrey, P. P. (1988) *Biochem. J.* **249**, 345–350.
- Yada, Y., Nagao, S., Okano, Y. & Nozawa, Y. (1989) *FEBS Lett.* **242**, 368–372.
- Yaish, P., Gazit, A., Gilon, C. & Levitzki, A. (1988) *Science* **242**, 933–935.
- Yamada, K., Iwahashi, K. & Kase, H. (1987) *Biochem. Biophys. Res. Commun.* **144**, 35–40.
- Yatomi, Y., Higashihara, M., Tanabe, A., Ohashi, T., Oka, H. & Kume, S. (1987) *Biochem. Biophys. Res. Commun.* **148**, 1025–1029.

Received 18 September 1989/8 January 1990; accepted 24 January 1990