The relationship between cytosolic Ca^{2+} , *sn*-1,2-diacylglycerol and inositol 1,4,5-trisphosphate elevation in platelet-activating-factor-stimulated rabbit platelets

Influence of protein kinase C on production of signal molecules

Christine T. MURPHY,* Moira ELMORE,† Stuart KELLIE† and John WESTWICK*‡

*Pharmacology, School of Pharmacy and Pharmacology, University of Bath, Bath BA2 7AY, U.K., and †Yamanouchi Research Institute (U.K.), Littlemore Hospital, Oxford OX4 4XN, U.K.

The temporal and dose–response relationships of platelet-activating-factor (PAF)-induced changes in the concentrations of cytosolic Ca^{2+} ($[\text{Ca}^{2+}]_i$), $\text{Ins}(1,4,5)\text{P}_3$ and 1,2-diacylglycerol (DAG) were examined. In addition, phosphorylation of protein kinase C (PKC) substrate (40–47 kDa protein) was determined. In high-dose PAF-activated platelets, all three signal molecules increased rapidly and transiently, with the peak $\text{Ins}(1,4,5)\text{P}_3$ concentration preceding maximal elevation of $[\text{Ca}^{2+}]_i$ by 5 s. In low-dose PAF-activated platelets there were large increases in $[\text{Ca}^{2+}]_i$ and dense-granule release, without any increase in $\text{Ins}(1,4,5)\text{P}_3$ and DAG or 40–47 kDa protein phosphorylation. Staurosporine, a non-specific PKC inhibitor, produced enhanced elevations in the concentrations of $\text{Ins}(1,4,5)\text{P}_3$, DAG and thromboxane B_2 , and the duration of the Ca^{2+} signal in platelets stimulated with a high dose, but not a low dose, of PAF. These results suggest there are both phospholipase C-dependent and -independent changes in Ca^{2+} homeostasis. Endogenously activated PKC regulates the formation of signal molecules.

INTRODUCTION

Receptor-activated hydrolysis of $\text{PtdIns}(4,5)\text{P}_2$ via the action of phospholipase C generates $\text{Ins}(1,4,5)\text{P}_3$ (Berridge & Irvine, 1984) and *sn*-1,2-diacylglycerol (DAG) (Rittenhouse-Simmons, 1979). Both of these products are thought to be intracellular signal molecules which regulate certain aspects of cell activation (Berridge, 1984, 1987).

Ca^{2+} mobilization from intracellular stores is induced in permeabilized platelets by $\text{Ins}(1,4,5)\text{P}_3$ (O'Rourke *et al.*, 1985; Brass & Joseph, 1985), whereas influx of Ca^{2+} across the plasma membrane via signal-molecule-operated Ca^{2+} channels may be regulated by $\text{Ins}(1,4,5)\text{P}_3$ working in concert with one of its phosphorylated products, $\text{Ins}(1,3,4,5)\text{P}_4$ (Irvine, 1989, 1990). The only well-characterized source of $\text{Ins}(1,4,5)\text{P}_3$ is the hydrolysis of $\text{PtdIns}(4,5)\text{P}_2$, whereas its removal is regulated either by phosphorylation to $\text{Ins}(1,3,4,5)\text{P}_4$ by $\text{Ins}(1,4,5)\text{P}_3$ kinase or by degradation to $\text{Ins}(1,4)\text{P}_2$ by 5-phosphomonoesterase (Batty *et al.*, 1985; Connolly *et al.*, 1987; Mitchell *et al.*, 1989).

The hydrolysis of (poly)phosphoinositides is probably the major source of DAG (Rittenhouse-Simmons, 1979), although an additional source may be via the action of phospholipase D on phosphatidylcholine (Pai *et al.*, 1988; Löffelholz, 1989). In platelets, however, the presence and role of phospholipase D have yet to be verified (Rubin, 1988). Receptor-activated DAG elevation is transient, probably owing to its phosphorylation by DAG kinase to phosphatidic acid (Tynes *et al.*, 1988) and its degradation by DAG lipase to fatty acid and glycerol (Bell *et al.*, 1979; Mahaevappa & Holub, 1986). This short-lived increase in DAG is thought to activate protein kinase C (PKC) (Nishizuka, 1984; Lapetina *et al.*, 1985), a family of ubiquitously distributed isoenzymes which have been implicated as having many different roles, including the regulation of cell activation (Nishizuka,

1984, 1988). The main substrate for PKC in platelets is a 40–47 kDa protein of unknown function (Kaibuchi *et al.*, 1983; Touqui *et al.*, 1986).

Little work has been carried out on intact platelet preparations to study the relationship between the signal molecules cytosolic Ca^{2+} , $\text{Ins}(1,4,5)\text{P}_3$ and DAG. In this study we have determined the temporal and dose–response relationships of platelet-activating-factor (PAF)-induced elevations in $[\text{Ca}^{2+}]_i$ to changes in the levels of $\text{Ins}(1,4,5)\text{P}_3$ and DAG in intact rabbit platelets. As an alternative experimental approach we have used the potent, although non-specific, PKC inhibitor staurosporine (Tamaoki *et al.*, 1986) to examine the role of endogenously activated PKC in the regulation of receptor-stimulated changes in the concentrations of $[\text{Ca}^{2+}]_i$, $\text{Ins}(1,4,5)\text{P}_3$ and DAG.

MATERIALS AND METHODS

Materials

Prostacyclin was generously provided by Dr. B. J. R. Whittle, Wellcome Laboratories, Beckenham, Kent, U.K. It was stored solid and dry at -20°C , and stock portions were dissolved in 0.5 M-Tris/HCl (pH 10.5) and were stored at a concentration of 40 $\mu\text{g}/40\ \mu\text{l}$ at -20°C . Immediately before use a portion was thawed and diluted as required in 0.01 M-Tris/HCl (pH 10.5), and then stored on ice. DAG assay reagents system (code RPN 200), $[\text{^3H}]\text{Ins}(1,4,5)\text{P}_3$ assay system (code TRK 1000), $[\text{^32P}]\text{ATP}$ (3 Ci/mmol), 5-hydroxy $[\text{^14C}]\text{tryptamine}$ (5-HT) creatinine sulphate (54 mCi/mmol, 50 $\mu\text{Ci}/\text{ml}$) and $[\text{^32P}]\text{P}_i$ (5 mCi/ml) were purchased from Amersham International, thin-layer plates (silica gel 60 HPTLC aluminium; 20 cm $\times$ 20 cm) were purchased from Merck, and X-ray film was from Fuji. Fura-2 AM (acetoxymethyl ester; cell-permeant) obtained from Molecular Probes, Eugene, OR, U.S.A. was stored dry at

Abbreviations used: DAG, 1,2-diacylglycerol; $[\text{Ca}^{2+}]_i$, cytosolic Ca^{2+} concn.; $[\text{Ca}^{2+}]_o$, extracellular Ca^{2+} concn.; PAF, platelet-activating factor; PKC, protein kinase C; HBT, Hepes-buffered Tyrode's solution; TxB_2 , thromboxane B_2 ; 5-HT, 5-hydroxytryptamine; PMA, phorbol 12-myristate 13-acetate.

‡ To whom correspondence and requests for reprints should be addressed.

–20 °C. A stock solution at a concentration of 5 mM in anhydrous dimethyl sulphoxide was also stored at –20 °C. PAF (1-*O*-octadecyl-2-acetyl-*sn*-glycero-3-phosphocholine) was supplied by Bachem (Bubendorf, Switzerland). A stock solution of 10 mM made up in chloroform/methanol (4:1, v/v) was stored at –20 °C, and working solutions (0.1 mM) were prepared in Ca^{2+} /Mg $^{2+}$ -free phosphate-buffered saline to which 2.5 mg of BSA/ml had been added. WEB 2086, a gift from Dr. H. Huer (Boehringer, Ingelheim, Germany), was dissolved in phosphate-buffered saline. Phorbol 12-myristate 13-acetate (PMA) was obtained from Sigma, dissolved in dry acetone and stored at –20 °C. Staurosporine was purchased from Fluka Chemicals, Glossop, U.K., dissolved in dimethyl sulphoxide and stored at –20 °C. Thromboxane B_2 (TxB_2) standard was obtained from Sigma, [^3H] TxB_2 (125 Ci/mmol, 50 nCi/ml) was from New England Nuclear, and anti-(rabbit TxB_2) antibody was generously supplied by Dr. F. Carey, ICI, Macclesfield, Cheshire, U.K.

Preparation of platelets

Washed rabbit platelets were prepared as described for human platelets by Poll & Westwick (1986), by a modification of an original method by Blackwell *et al.* (1982). The resulting platelet pellet from this preparation was suspended in Hepes-buffered Tyrode's (HBT) solution (10 mM-Hepes, 145 mM-NaCl, 5 mM-KCl, 1 mM-MgCl $_2$, 0.5 mM-Na $_2$ HPO $_4$, 5.5 mM-glucose and 0.25% BSA, pH 7.4) and the platelet count was adjusted.

Protein phosphorylation

Platelets (8×10^9 /ml) were incubated for 2 h with 200 μCi of [^{32}P]P $_i$ /ml of platelets, washed in HBT, and the platelet count was adjusted to 10^9 /ml. Batches of platelets (300 μl ; 10^9 /ml) were equilibrated at 37 °C in the presence of 1 mM-[Ca^{2+}] $_e$. Platelets were incubated with a dose range of PAF, and activation was terminated with 300 μl of 2% Laemmli (1970) SDS reducing gel-sample buffer (100 °C) at 30 s after PAF addition. The samples were then boiled for 5 min, and centrifuged at 8000 *g* for 2 min to remove any particles. Proteins (100 μg /track) were separated by SDS/10%-polyacrylamide-gel electrophoresis; protein bands and molecular markers were stained with Coomassie Blue, and [^{32}P]phosphorylated bands were observed by autoradiography. The phosphorylated bands were excised and quantified by liquid-scintillation counting.

Measurement of DAG production

Platelet DAG production was determined with the DAG assay reagents system (Amersham International, RPN 200), which used an adaptation of the method described by Preiss *et al.* (1986). Briefly, 1 ml portions of platelets (10^9 /ml) were dispensed into cuvettes and the [Ca^{2+}] $_e$ was adjusted to 1 mM. Platelets were continually stirred and allowed to equilibrate to 37 °C before addition of drug/vehicle control. All drugs were incubated for 2 min before addition of PAF. DAG production was stopped at pre-determined times by removing 0.8 ml into 3 ml of ice-cold methanol/chloroform (2:1, v/v), and samples were extracted by a modification of the method of Bligh & Dyer (1959). The assay employed was a radio-enzymic assay using *Escherichia coli* DAG kinase and defined mixed-micelle conditions to solubilize the DAG present and allow its quantitative conversion into [^{32}P]phosphatidic acid in the presence of [^{32}P]ATP. The sensitivity of the assay was < 10 pmol, and the working range was 31–1000 pmol/tube.

Determination of $\text{Ins}(1,4,5)\text{P}_3$ production

Experiments were carried out as described for DAG determination. The reaction was terminated and $\text{Ins}(1,4,5)\text{P}_3$ was extracted by mixing the cell suspension with 0.2 vol of ice-cold

20% (v/v) HClO $_4$ and after 20 min sedimenting the proteins by centrifugation. The supernatant was then titrated to pH 7.5 with 1.5 M-KOH containing 60 mM-Hepes buffer and Universal indicator. The $\text{Ins}(1,4,5)\text{P}_3$ present in the samples was determined by use of a binding assay which employed unlabelled $\text{Ins}(1,4,5)\text{P}_3$, [^3H] $\text{Ins}(1,4,5)\text{P}_3$ and a bovine adrenal-membrane preparation (Challiss *et al.*, 1988). The sensitivity of the assay was < 0.2 pmol, and the working range of $\text{Ins}(1,4,5)\text{P}_3$ detected by this assay was 0.2–25 pmol/tube.

Determination of [Ca^{2+}] $_i$

Platelets were loaded with 2.5 μM -fura-2 AM for 30 min at 37 °C, the platelet suspension was then washed in HBT to remove excess fura-2 AM, and the platelet count adjusted to 2×10^8 /ml. To measure the changes in [Ca^{2+}] $_i$, fluorescence readings were recorded at 37 °C with an Aminco Bowman spectrophotofluorimeter (excitation at 339 nm; emission at 500 nm; 4 nm slit width) fitted with a thermostatically regulated cuvette compartment holder with stirring attachment. Samples (1 ml) of platelets were dispensed into cuvettes, and [Ca^{2+}] $_e$ was adjusted to 1 mM. The Ca^{2+} -fura-2 fluorescence was calibrated as we have previously described by using EGTA quench (Ward & Westwick, 1988). Platelets were equilibrated to 37 °C before the addition of drugs or drug vehicles, which were incubated for 2 min before addition of PAF.

Measurement of percentage [^{14}C]5-HT release

Platelets (8×10^8 /ml) were incubated with 200 nCi of [^{14}C]5-HT/ml, then washed in HBT, and the count was adjusted to 2×10^8 platelets/ml. [Ca^{2+}] $_e$ was adjusted to 1 mM, and platelets were equilibrated to 37 °C before addition of a drug or vehicle control. To determine 5-HT release from the platelets, sub-samples of platelets (400 μl) were withdrawn 3 min after addition of the agonist into ice-cooled tubes containing 40 μl of 100 mM-EDTA and 30 μM -indomethacin. The samples were centrifuged at 12000 *g* for 2 min and $2 \times 100 \mu\text{l}$ samples of the supernatant were removed and the amount of [^{14}C]5-HT was determined by liquid-scintillation spectroscopy. Percentage 5-HT release was determined as previously described (Holmsen *et al.*, 1981).

Measurement of TxB_2 generation

TxB_2 generation was determined in 1 ml samples of platelets (2×10^8 /ml), with [Ca^{2+}] $_e$ adjusted to 1 mM. Platelets were equilibrated to 37 °C before addition of drug or drug vehicle, which was added 2 min before PAF. To terminate TxB_2 generation, sub-samples of platelets (400 μl) were withdrawn at pre-determined times (3 min unless otherwise stated) into 40 μl of ice-cold 100 mM-EDTA/30 μM -indomethacin. The samples were centrifuged, the supernatant was removed and the TxB_2 content determined by radioimmunoassay (Johnston *et al.*, 1984).

Statistics

Data in the Figures are means $\pm$ S.E.M. of at least three experiments each performed in either duplicate or triplicate. To determine the levels of significance between sets of data, a paired Student's *t* test was employed. The levels of significance are denoted by * P < 0.05, ** P < 0.01, *** P < 0.005. The Bonferroni correction was applied where necessary (Wallenstein *et al.*, 1980).

RESULTS

PAF-induced elevation of [Ca^{2+}] $_i$ and concentrations of $\text{Ins}(1,4,5)\text{P}_3$ and DAG

Addition of 300 nM-PAF induced a rapid elevation of platelet [Ca^{2+}] $_i$ from a basal concentration of 152 ± 11 nM ($n = 30$) to a

Fig. 1. Time courses for the production of DAG, Ins(1,4,5)P₃ and [Ca²⁺]_i in 300 nM-PAF-stimulated platelets

DAG production induced by 300 nM-PAF is shown in (a), and [Ca²⁺]_i elevation (○) together with Ins(1,4,5)P₃ elevation (■) are shown in (b). The values in all Figures were obtained by subtracting the basal pre-PAF value from those obtained at the stated times after administration of 300 nM-PAF. The values are means ± S.E.M. of at least three experiments. The S.E.M. bars are absent when symbols are larger than the S.E.M.

Table 1. Effect of 2 min pre-treatment with the PAF receptor antagonist WEB 2086 on PAF-induced Ins(1,4,5)P₃ and DAG generation

Treatment	Generation (% of control)	
	Ins(1,4,5)P ₃ *	DAG†
Vehicle + 300 nM-PAF	100	100
30 nM-WEB 2086 + 300 nM-PAF	24.0 ± 14	22.5 ± 16
300 nM-WEB 2086 + 300 nM-PAF	4.7 ± 3.2	0

* Measured 5 s after PAF addition.
† Measured 10 s after PAF addition.

peak elevation of 1023 ± 72 nM at 7.3 ± 0.6 s (n = 13) after PAF addition. This elevation was transient, and quickly declined (Fig. 1). Similarly, in the same platelet preparations 300 nM-PAF induced a rapid elevation in the concentration of Ins(1,4,5)P₃, with a peak increase at 2 s of 38.9 ± 2.2 pmol/10⁹ platelets above a basal level of 9.77 ± 1.72 pmol/10⁹ platelets (Fig. 1). This elevation rapidly declined to basal levels by 15 s after PAF, followed by a gradual increase at 30 and 60 s after PAF.

Superimposition of the Ins(1,4,5)P₃ and [Ca²⁺]_i time curves demonstrates that the Ins(1,4,5)P₃ peak clearly precedes the peak [Ca²⁺]_i by approx. 5 s.

Platelets had a basal concentration of 117 ± 3 pmol of DAG/10⁹ platelets. After addition of 300 nM-PAF, there was a rapid and transient increase in the platelet DAG concentration, to a peak at 10 s with a value of 280 ± 52 pmol/10⁹ platelets (n = 9) above basal. After 10 s the concentration of DAG rapidly declined, and by 15 s reached a level above basal, at which it remained for the duration of the time course (60 s) (Fig. 1).

Fig. 2. Effect of increasing concentrations of PAF on production of DAG and Ins(1,4,5)P₃ (a) and [Ca²⁺]_i elevation and 5-HT release (b)

DAG incubations were terminated at 10 s after PAF, and those for Ins(1,4,5)P₃ at 5 s. 5-HT release was terminated at 3 min after activation and [Ca²⁺]_i is the peak elevation after stimulation. The values were obtained by subtracting the basal pre-PAF value from those obtained after addition of PAF. The values are means ± S.E.M. of at least three experiments.

Fig. 3. Effect of replacing 1 mM-Ca²⁺ with 2 mM-EGTA on PAF-induced [Ca²⁺]_i elevation

[Ca²⁺]_i is the value obtained by subtracting the basal pre-PAF value from the peak post-PAF value. Each value represents the mean ± S.E.M. of at least three experiments each performed in triplicate. Key: ○, control; ●, +2 mM-EGTA.

Addition of PAF vehicle did not produce changes in the basal concentrations of [Ca²⁺]_i, Ins(1,4,5)P₃ or DAG (results not shown).

Table 1 demonstrates that pre-treatment of the platelets with the PAF-receptor antagonist WEB 2086 (300 nM) inhibited to the same degree PAF-induced elevation of Ins(1,4,5)P₃ and DAG.

Dose-response relationship of PAF-induced platelet activation

The relationship of the concentrations of the three putative signal molecules, [Ca²⁺]_i, Ins(1,4,5)P₃ and DAG, to the release of 5-HT was determined (Fig. 2).

PAF is a powerful dense-granule releasing agent, and the dose-response relationship is shown in Fig. 2(b). The higher

Fig. 4. Effect of increasing concentrations of PAF on phosphorylation of the 40 kDa substrate of PKC

(a) Autoradiograph showing phosphorylation of the 40 kDa substrate of PKC by PAF vehicle (track 1) or by 0.3, 3, 30 and 300 nM-PAF respectively (tracks 2-5) and with 100 nM-PMA (track 6). The phosphorylated PKC substrate band was excised and subjected to scintillation counting, and the results are given in (b) as a percentage of phosphorylation with PAF vehicle.

doses of PAF, 30 and 300 nM, which are maximal and supra-maximal for 5-HT release, produced highly significant ($P < 0.005$) elevations in $[Ca^{2+}]_i$, in addition to a rise in the concentrations of $Ins(1,4,5)P_3$ (measured at 5 s) and DAG (measured at 10 s) (Fig. 2a). However, a lower concentration of PAF, such as 3 nM, which induced approx. 60% 5-HT release and a 400 nM increase above the basal $[Ca^{2+}]_i$, induced no detectable increase in either $Ins(1,4,5)P_3$ or DAG. Replacing $[Ca^{2+}]_i$ with the Ca^{2+} chelator EGTA inhibited $[Ca^{2+}]_i$ elevation at all concentrations of PAF tested (Fig. 3). At the higher concentrations of PAF, 30 and 300 nM, and to a lesser extent at 3 nM, $[Ca^{2+}]_i$ elevation was due to two components, the major one being dependent on $[Ca^{2+}]_e$, whereas the minor one was independent of $[Ca^{2+}]_e$. At the lower concentration of 0.3 nM-PAF, however, $[Ca^{2+}]_i$ elevation was completely dependent on the presence of $[Ca^{2+}]_e$. In relation to the findings for DAG, activation of PKC, measured as phosphorylation of its 40 kDa substrate, was increased dose-dependently with increasing concentrations of PAF; 3 nM-PAF induced some activation of PKC after 30 s, but, again in agreement with the findings for DAG, no phosphorylation with 0.3 nM-PAF was detected (Fig. 4).

These results suggest that there is a good correlation between both $PtdInsP_2$ hydrolysis/ Ca^{2+} mobilization and granule release at higher doses, but not at lower concentrations of PAF.

Fig. 5. Effect of PKC activation or inhibition on generation of DAG and $Ins(1,4,5)P_3$ induced by 3 nM- and 300 nM-PAF

PKC was pre-activated with 100 nM-PMA or pre-inhibited with 1 μ M-staurosporine. DAG incubations (b) were terminated 10 s after the addition of PAF, and $Ins(1,4,5)P_3$ incubations (a) were terminated at 5 s. Each value represents the mean $\pm$ S.E.M. of at least three experiments each performed in duplicate. Key: $\square$, control; $\square$ with diagonal lines, 1 μ M-staurosporine; $\square$ with horizontal lines, 100 nM-PMA.

Relationship between PKC and PAF-induced platelet activation

To explore the role of endogenous activators of PKC on the regulation of PAF-induced formation of $Ins(1,4,5)P_3$, DAG, TxB_2 and changes in Ca^{2+} homeostasis, the effect of the potent PKC inhibitor staurosporine on these parameters was examined: 1 μ M-staurosporine was used, as we have previously demonstrated that this concentration inhibits at least 70% of PKC activation (Murphy & Westwick, 1990).

$Ins(1,4,5)P_3$ and DAG. Pretreatment of the platelets with 1 μ M-staurosporine did not modify the basal concentrations of either $Ins(1,4,5)P_3$ or DAG (Fig. 5). Thus PKC does not exert a tonic control over these parameters in the unstimulated platelets. The effect of staurosporine on PAF-stimulated formation of $Ins(1,4,5)P_3$ and DAG was dependent on the concentration of PAF employed. Staurosporine produced a 2- and 3-fold enhancement of $Ins(1,4,5)P_3$ and DAG formation respectively in response to 300 nM-PAF (Fig. 5). When 3 nM-PAF was used, which alone failed to produce an increase in $Ins(1,4,5)P_3$ or DAG over the basal levels, pretreatment with staurosporine did not produce any detectable increase over this.

Pretreatment of the platelets with the PKC activator PMA (100 nM) produced a significant decrease in basal and PAF-stimulated levels of DAG (Fig. 5).

Ca^{2+} homeostasis. Pretreatment of platelets with 1 μ M-staurosporine did not modify the basal $[Ca^{2+}]_i$ of platelets (results not shown). In addition, preincubation of platelets with staurosporine had no significant effect on the rate of elevation or the maximum elevation of $[Ca^{2+}]_i$, stimulated with either 3 nM- or 300 nM-PAF (Fig. 6). However, the duration of the Ca^{2+} signal, measured as the time taken to return to half-maximum elevation above basal ($t_{1/2}$), was significantly potentiated by staurosporine for both concentrations of PAF (Fig. 6). The increase in $t_{1/2}$ in platelets stimulated with 3 nM-PAF was only 30%, in contrast

Fig. 6. Effect of staurosporine on PAF-induced $[Ca^{2+}]_i$ elevation

Effect of $1 \mu M$ -staurosporine on the peak elevation of $[Ca^{2+}]_i$ or on the $t_{1/2}$ (time for decay of elevated $[Ca^{2+}]_i$ to half-maximal elevation over basal) in platelets stimulated with 3 nM- or 300 nM-PAF. (a) Representative trace of fluorescence output transformed to $[Ca^{2+}]_i$ from fura-2-loaded platelets, demonstrating the concepts of peak $[Ca^{2+}]_i$ and $t_{1/2}$. (b) Effect of staurosporine on maximal $[Ca^{2+}]_i$ elevation. (c) Effect of staurosporine on the $t_{1/2}$. $[Ca^{2+}]_i$ is the value obtained by subtracting the basal pre-PAF value from the peak post-PAF value. Each value represents the mean $\pm$ S.E.M. of at least three experiments each performed in triplicate. Key: $\square$, control (C); $\blacksquare$, staurosporine (SS).

Fig. 7. Effect of $1 \mu M$ -staurosporine on PAF-induced TxB₂ generation

TxB₂ generation was determined 3 min after addition of PAF. The points represent means $\pm$ S.E.M. of three experiments each performed in triplicate. Key: $\square$, control; $\bullet$, staurosporine.

with the 200% increase in $t_{1/2}$ observed in 300 nM-PAF-stimulated platelets.

TxB₂ formation. Only trace amounts of TxB₂ (< 1 pmol/ml) were detected after platelet activation with either 0.3 nM- or 3 nM-PAF, and these levels were not affected by staurosporine (Fig. 7). However, at higher concentrations of PAF (30–300 nM) there was a dose-dependent increase in the TxB₂ generated, and this was potentiated at least 2-fold with staurosporine (Fig. 7).

To determine whether the staurosporine-enhanced TxA₂

formation was responsible for the increased DAG formation (Fig. 5), the effect of indomethacin on this system was examined. Pretreatment of the platelets with $2.8 \mu M$ -indomethacin, which caused $> 99\%$ inhibition of TxB₂ formation, had no significant ($P > 0.05$) effect on basal, PAF-induced or staurosporine-enhanced levels of DAG (results not shown).

DISCUSSION

We have examined the relationship between the three putative signal molecules, namely Ins(1,4,5)P₃, DAG and $[Ca^{2+}]_i$, in PAF-stimulated rabbit platelets, in both the absence and the presence of an inhibitor of PKC.

A very rapid and transient elevation of all three signal molecules was induced by the addition of a high concentration of PAF. The concentration of Ins(1,4,5)P₃ reached a peak at 2 s after PAF addition, which preceded the peak concentration of $[Ca^{2+}]_i$ by 5 s. In addition, the peak concentration of Ins(1,4,5)P₃ was 45 pmol/10⁹ platelets, which is equivalent to $4.5 \mu M$ (assuming that 10⁹ platelets have a cytosolic volume of 1 μl ; Rittenhouse & Sasson, 1985), close to the $5 \mu M$ -Ins(1,4,5)P₃ that is required for maximum release of ⁴⁵Ca²⁺ from permeabilized human platelets (Brass & Joseph, 1985) and membrane vesicles (O'Rourke *et al.*, 1985). Thus our observations in intact platelets, using high concentrations of PAF, provide good support for the proposal that Ins(1,4,5)P₃ is responsible for mobilizing intracellular Ca²⁺ (Berridge & Irvine, 1984).

The time course and the amount of DAG formed, compared with Ins(1,4,5)P₃, suggests that PtdInsP₂ hydrolysis may not be the only source of DAG. Possible additional sources include the hydrolysis of other phosphoinositides or the action of phospholipase D on phosphatidylcholine (Loffelholz, 1989). Whatever the source of DAG, the 3-fold increase over basal reported in the present study with PAF-stimulated rabbit platelets corresponds well to the 2–3-fold DAG elevation found in thrombin-stimulated human platelets, when the same assay for DAG was used (Preiss *et al.*, 1986). The parallel inhibition of PAF-induced elevations of Ins(1,4,5)P₃ and DAG by WEB 2086 (Casals-Stenzel *et al.*, 1986) indicates that these events are closely coupled to a single class of PAF receptor.

The higher concentrations of PAF (30 and 300 nM) produced dose-related increases in the amounts of $[Ca^{2+}]_i$, Ins(1,4,5)P₃, DAG, 40 kDa phosphorylation and granule release. However, sub-maximal concentrations of PAF (0.3 and 3 nM), which caused considerable dense-granule release and elevation of $[Ca^{2+}]_i$, failed to induce a detectable increase over basal in the concentration of either Ins(1,4,5)P₃ or DAG. Thus, in platelets activated with sub-maximal levels of PAF, neither the Ins(1,4,5)P₃-dependent mobilization of Ca²⁺ from intracellular stores nor the DAG-dependent activation of PKC would be expected. The lack of $[Ca^{2+}]_i$ elevation in the absence of $[Ca^{2+}]_e$ at low levels of PAF, together with the decreased phosphorylation of PKC substrate with sub-maximal levels of PAF, support this.

These findings have several implications. Firstly, at higher concentrations of PAF, there is a good correlation between products of PtdInsP₂ hydrolysis and platelet activation, but at the lower concentrations of PAF, platelet activation, including Ca²⁺ mobilization, occurs independently of PtdInsP₂ hydrolysis. Indeed, in other cell types a lack of correlation between PKC activation and functional response, or Ins(1,4,5)P₃ elevation and $[Ca^{2+}]_i$ elevation, has been found. A lack of correlation between DAG accumulation and superoxide-anion production after stimulation of neutrophils with either concanavalin A (Rider & Nield, 1987) or *N*-formylmethionyl-leucyl-phenylalanine (Koenderman *et al.*, 1989) has been observed. In vascular smooth-muscle cells stimulated with PAF, the time to peak

$[Ca^{2+}]_i$ elevation is reached before any elevation in $Ins(1,4,5)P_3$ (Schwertschlag & Whorton, 1988), and it has been suggested that other mechanisms are responsible for regulating PAF-induced Ca^{2+} mobilization, namely a receptor-operated Ca^{2+} channel independent of phosphatidylinositol polyphosphate hydrolysis. Similarly, in ADP-stimulated platelets Ca^{2+} entry was found clearly to precede internal discharge, and it was suggested that receptor occupancy is closely coupled to the opening of plasma-membrane bivalent-cation channels (Sage *et al.*, 1989). Furthermore, in hepatocytes the formation of $Ins(1,4,5)P_3$ has been found to require a higher concentration of receptor-operated hormone than does $[Ca^{2+}]_i$ elevation (Lynch *et al.*, 1989). This finding has been explained as either $Ins(1,4,5)P_3$ elevation and $[Ca^{2+}]_i$ elevation not being causally related, or by the presence of spare receptors for agonists that mobilize Ca^{2+} . Moreover, PAF has been shown to increase $[Ca^{2+}]_i$ in neutrophils where phospholipase C activation, and therefore $Ins(1,4,5)P_3$ production, have been inhibited by pertussis toxin (Naccache *et al.*, 1985).

To determine the role of PKC in the regulation of early post-receptor events, we used the potent, although non-specific, PKC inhibitor staurosporine (Tamaoki *et al.*, 1986; Watson *et al.*, 1987). A concentration of 1 μM -staurosporine was selected, as we had previously shown that this concentration inhibited, by at least 70%, 300 nM-PAF-induced PKC-substrate phosphorylation (Murphy & Westwick, 1990). Both DAG and $Ins(1,4,5)P_3$ production induced by 300 nM-PAF were significantly potentiated by PKC inhibition; however, there was no effect on $Ins(1,4,5)P_3$ or DAG levels in platelets stimulated with 3 nM-PAF. Previous studies, using phorbol esters or membrane-permeant DAG to activate PKC before agonist or fluoride stimulation in platelets, showed that some functional responses were inhibited by pre-activation of PKC (Poll & Westwick, 1986; Poll *et al.*, 1986; Krishnamurthi *et al.*, 1986). This led to the theory that, when activated, PKC caused a negative feedback, inhibiting generation of both DAG and $Ins(1,4,5)P_3$ at the level of production (at either phospholipase C or a controlling G-protein) (Watson & Lapetina, 1985; Zavoico *et al.*, 1985). The level of $Ins(1,4,5)P_3$ was further suppressed as activation of PKC increased its metabolism to $Ins(1,4)P_2$ (Molina y Vedia & Lapetina, 1986; Connolly *et al.*, 1986).

We suggest that in our system, where PKC is activated by higher concentrations of PAF, DAG and $Ins(1,4,5)P_3$ levels are depressed by negative feedback. However, when PKC is inhibited the negative feedback is removed and a full elevation of DAG and $Ins(1,4,5)P_3$ induced by 300 nM-PAF stimulation results. At lower concentrations of PAF no negative feedback would be exerted by PKC, and therefore the inhibition of PKC would, as observed, have no effect. However, although the findings suggest that PKC is exerting its effect on the production of $Ins(1,4,5)P_3$ and DAG, as they are both formed from the hydrolysis of $PtdInsP_2$ by phospholipase C, the findings do not dismiss the possibility that PKC may also have a role in the removal of $Ins(1,4,5)P_3$ and DAG (King & Rittenhouse, 1989). Another interesting point from these findings is that the activator of PKC, namely DAG, appears to be self-regulating.

We also attempted to correlate the effect of PKC inhibition on generation of DAG or $Ins(1,4,5)P_3$ with the effect on the functional responses of $[Ca^{2+}]_i$ elevation and TxB_2 generation. Firstly, staurosporine had no significant effect on either 3 nM- or 300 nM-PAF-induced peak $[Ca^{2+}]_i$ elevation, despite an increase in $Ins(1,4,5)P_3$ on stimulation by 300 nM-PAF. This could be due to the limits of detection of $[Ca^{2+}]_i$ elevation being reached after stimulation with 300 nM-PAF, and therefore any further increase in $[Ca^{2+}]_i$ being indistinguishable, or that maximal intracellular Ca^{2+} mobilization had already been achieved. There was no

increase in $Ins(1,4,5)P_3$ at 3 nM-PAF, however, and although it would have been possible to detect an increase in $[Ca^{2+}]_i$ at this concentration of PAF, no significant increase was observed. However, when investigating the $t_{1/2}$ of the decay of $[Ca^{2+}]_i$ it was found that PKC inhibition produced a 200% increase in the $t_{1/2}$ of elevated $[Ca^{2+}]_i$ in 300 nM-PAF-stimulated platelets, but only a 30% increase in $t_{1/2}$ in platelets stimulated with 3 nM-PAF. These findings are in agreement with the work of Watson & Hambleton (1989), where an increase in $t_{1/2}$ of $[Ca^{2+}]_i$ decay was observed in thrombin-stimulated platelets pretreated with 1 μM -staurosporine, despite no evidence of a potentiation of $Ins(1,4,5)P_3$ (Watson *et al.*, 1987).

These results taken together also suggest that PKC may be acting to restore the elevated $[Ca^{2+}]_i$ to basal levels, as was shown by Poll & Westwick (1986) and Pollock *et al.* (1987) in human platelets. Alternatively, our findings are in agreement with those of King & Rittenhouse (1989), in respect of PKC inhibition potentiating $Ins(1,4,5)P_3$ levels, although unlike us they found that this caused an increase in $[Ca^{2+}]_i$ elevation. Moreover, King & Rittenhouse (1989) found no potentiation in thrombin-induced DAG levels by 10 μM -staurosporine. This is interesting, because Bishop *et al.* (1989) found that, with a high concentration of the PKC inhibitor sphingosine, the DAG elevation induced by thrombin was abolished, but at lower concentrations of sphingosine the DAG level was potentiated, and they attributed the inhibition by sphingosine to an inhibition of proximal portions of the signal-transduction pathway at this higher concentration of inhibitor. Therefore it may be that the concentration of 10 μM -staurosporine used in the study by King & Rittenhouse (1989), which is 10-fold higher than that used in our study, has masked any potentiatory response which may have been observed at a lower concentration of staurosporine.

We also examined the effect of staurosporine on PAF-induced TxB_2 generation. At lower concentrations of PAF (0.3–3 nM), only trace amounts of TxB_2 were detected, but at higher PAF concentrations (30 nM and 300 nM) TxB_2 was dose-dependently increased, and this increase was potentiated by inhibition of PKC. Earlier studies have shown that pre-activation of PKC causes an inhibition of agonist-induced TxB_2 generation (Murphy & Westwick, 1989) and fluoride-induced TxB_2 generation (Poll *et al.*, 1986). Inhibition of PKC, and therefore inhibition of the negative feedback exerted by activated PKC, would indeed predict a potentiation in TxB_2 generation. The increase in TxB_2 induced by PKC inhibition may be attributed to a number of events. Firstly, phospholipase A_2 is Ca^{2+} -dependent, and it may be that the sustained duration of elevation of $[Ca^{2+}]_i$ induced by PKC inhibition is increasing the activation of this enzyme and thus causing the observed potentiation in TxB_2 . Alternatively, the increased production of DAG could provide, via DAG lipase, increased levels of arachidonic acid and ultimately TxB_2 production (conversely, the use of indomethacin provided evidence that the increase in TxA_2 was not responsible for the increase in DAG production). Finally, it is also possible that PKC is exerting a direct effect on phospholipase A_2 or a closely coupled GTP-binding protein (Silk *et al.*, 1990) or that it is affecting TxB_2 production by modulating a family of proteins called lipocortins which, if present, may be responsible for inhibiting phospholipase A_2 activity (Touqui *et al.*, 1986).

Several conclusions can be drawn from the present work. The relationship between the concentrations of the signal molecules and the platelet functional responses is very dependent on the dose of PAF employed. In high-dose-PAF-activated platelets we have shown that the elevation of $Ins(1,4,5)P_3$ clearly precedes the $[Ca^{2+}]_i$ elevation, supporting the proposal that $Ins(1,4,5)P_3$ is responsible for this mobilization of intracellular Ca^{2+} stores. In contrast, in low-dose-activated platelets, elevation of $[Ca^{2+}]_i$ and

granule release occurs in the absence of detectable increases in the concentration of $\text{Ins}(1,4,5)\text{P}_3$ or DAG, as well as an absence of Ca^{2+} mobilization from intracellular stores or PKC-substrate phosphorylation.

These results suggest that in platelet activation induced by low concentrations of agonist, mechanisms other than polyphosphoinositide bisphosphate hydrolysis are utilized for platelet activation. The existence of these separate mechanisms is supported by experiments with an inhibitor of PKC, which results in a marked potentiation of DAG, $\text{Ins}(1,4,5)\text{P}_3$ and TxB_2 production as well as a protracted Ca^{2+} signal induced to a much greater extent by the higher doses of PAF. Finally, the results demonstrate that endogenously activated PKC operates as a powerful negative-feedback regulator of signal molecules.

C.T.M. and J.W. are grateful to the British Heart Foundation for support.

REFERENCES

- Batty, I. R., Nahorski, S. R. & Irvine, R. F. (1985) *Biochem. J.* **232**, 211–215
- Bell, R. L., Kennerly, D. A., Stanford, N. & Majerus, P. W. (1979) *Proc. Natl. Acad. Sci. U.S.A.* **76**, 3238–3241
- Berridge, M. J. (1984) *Biochem. J.* **220**, 345–360
- Berridge, M. J. (1987) *Annu. Rev. Biochem.* **56**, 159–193
- Berridge, M. J. & Irvine, R. F. (1984) *Nature (London)* **312**, 315–321
- Bishop, W. R., August, J. & Petrin, J. (1989) *Proc. Int. Washington Symp. 9th Biol. Cell. Transduction Signals* P234
- Blackwell, G. J., Radomski, M., Margas, J. R. & Moncada, S. (1982) *Biochim. Biophys. Acta* **718**, 60–65
- Bligh, E. G. & Dyer, W. J. (1959) *Can. J. Biochem. Physiol.* **37**, 911–917
- Brass, L. F. & Joseph, S. K. (1985) *J. Biol. Chem.* **260**, 15172–15179
- Casals-Stenzel, J., Muacevic, G. & Weber, K.-H. (1986) *Naunyn-Schmiedeberg's Arch. Pharmacol.* **334**, Suppl., R44175
- Challiss, R. A. J., Batty, I. H. & Nahorski, S. R. (1988) *Biochem. Biophys. Res. Commun.* **157**, 684–691
- Connolly, T. M., Lawing, W. J., Jr. & Majerus, P. W. (1986) *Cell* **46**, 951–958
- Connolly, T. M., Bansal, V. S., Bross, T. E., Irvine, R. F. & Majerus, P. W. (1987) *J. Biol. Chem.* **262**, 2146–2149
- Holmsen, H., Dangelmaier, C. A. & Holmsen, K.-H. (1981) *J. Biol. Chem.* **256**, 9393–9396
- Irvine, R. F. (1989) *Biochem. Soc. Trans.* **17**, 6–9
- Irvine, R. F. (1990) *FEBS Lett.* **263**, 5–9
- Johnston, C., Carey, F., Forster, R. A. & Haworth, D. (1984) *Clin. Sci.* **67**, 633–638
- Kaibuchi, K., Takai, Y., Sawamura, M., Hoshijima, M., Fujikura, T. & Nishizuka, Y. (1983) *J. Biol. Chem.* **258**, 6701–6704
- King, W. G. & Rittenhouse, S. E. (1989) *J. Biol. Chem.* **264**, 6070–6074
- Koenderman, L., Tool, A., Roos, D. & Verhoeven, A. J. (1989) *FEBS Lett.* **243**, 399–403
- Krishnamurthi, S., Joseph, S. & Kakkar, V. V. (1986) *Biochem. J.* **238**, 193–199
- Laemmli, U. K. (1970) *Nature (London)* **227**, 680–685
- Lapetina, E. G., Reep, B., Ganong, B. R. & Bell, R. M. (1985) *J. Biol. Chem.* **260**, 1358–1361
- Löffelholz, K. (1989) *Biochem. Pharmacol.* **38**, 1543–1549
- Lynch, C. J., Blackmore, P. F., Charest, R. & Exton, J. H. (1989) *Mol. Pharmacol.* **28**, 93–99
- Mahaevappa, V. G. & Holub, B. J. (1986) *Biochem. Biophys. Res. Commun.* **134**, 1327–1333
- Mitchell, C. A., Connolly, T. M. & Majerus, P. W. (1989) *J. Biol. Chem.* **264**, 8873–8877
- Molina y Vedia, L. M. & Lapetina, E. G. (1986) *J. Biol. Chem.* **261**, 10493–10495
- Murphy, C. T. & Westwick, J. (1989) *Br. J. Pharmacol.* **96**, 70P
- Murphy, C. T. & Westwick, J. (1990) *J. Lipid Mediators* **2**, 205P
- Naccache, P. H., Molski, M. M., Volpi, M., Becker, E. L. & Sha'afi, R. I. (1985) *Biochem. Biophys. Res. Commun.* **130**, 677–684
- Nishizuka, Y. (1984) *Nature (London)* **308**, 693–698
- Nishizuka, Y. (1988) *Nature (London)* **334**, 661–665
- O'Rourke, F. A., Halenda, S. P., Zavoico, G. B. & Feinstein, M. B. (1985) *J. Biol. Chem.* **260**, 956–962
- Pai, J.-K., Seigel, M. I., Egan, R. W. & Billah, M. M. (1988) *J. Biol. Chem.* **263**, 12472–12477
- Poll, C. & Westwick, J. (1986) *Biochim. Biophys. Acta* **886**, 434–440
- Poll, C., Kyrle, P. & Westwick, J. (1986) *Biochem. Biophys. Res. Commun.* **136**, 381–389
- Pollock, W. K., Sage, S. O. & Rink, T. J. (1987) *FEBS Lett.* **210**, 132–136
- Preiss, J., Loomis, C. R., Bishop, W. R., Stein, R., Niedel, J. E. & Bell, R. M. (1986) *J. Biol. Chem.* **261**, 8597–8600
- Rider, L. G. & Niedel, J. E. (1987) *J. Biol. Chem.* **262**, 5603–5608
- Rittenhouse, S. E. & Sasson, J. P. (1985) *J. Biol. Chem.* **260**, 8657–8660
- Rittenhouse-Simmons, S. (1979) *J. Clin. Invest.* **63**, 580–587
- Rubin, R. (1988) *Biochem. Biophys. Res. Commun.* **156**, 1090–1096
- Sage, S. O., Merritt, J. E., Hallam, T. J. & Rink, T. J. (1989) *Biochem. J.* **258**, 923–926
- Schwertschlag, U. S. & Whorton, A. R. (1988) *J. Biol. Chem.* **263**, 13791–13796
- Silk, S. T., Clejan, S. & Witkom, K. (1990) *J. Biol. Chem.* **264**, 21466–21469
- Tamaoki, T., Hisayo, N., Takahashi, I., Kato, Y., Makoto, M. & Tomita, F. (1986) *Biochem. Biophys. Res. Commun.* **135**, 397–402
- Touqui, L., Rothhut, B., Shaw, A. M., Fraolin, A., Vargaftig, B. B. & Russo-Marie, F. (1986) *Nature (London)* **321**, 177–180
- Tysnes, O.-B., Berhoeven, A. J. M. & Holmsen, H. (1988) *Eur. J. Biochem.* **174**, 75–79
- Wallenstein, S., Zucker, C. L. & Fleiss, J. L. (1980) *Circ. Res.* **47**, 1–9
- Ward, S. & Westwick, J. (1988) *Br. J. Pharmacol.* **93**, 769–774
- 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., McNally, J., Shipman, L. J. & Godfrey, P. P. (1987) *Biochem. J.* **249**, 345–350
- Zavoico, G. B., Halenda, S. P., Sha'afi, R. I. & Feinstein, M. B. (1985) *Proc. Natl. Acad. Sci. U.S.A.* **82**, 3859–3862

Received 4 December 1990/15 March 1991; accepted 2 April 1991