Role of type 1 and type 2A phosphatases in signal transduction of platelet-activating-factor-stimulated rabbit platelets

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Calyculin A, the potent inhibitor of type 1 (PP1) and type 2A (PP2A) phosphatases, has been employed in order to investigate the role of endogenously activated PP1/PP2A in the signal-transduction pathway of platelet-activating-factor (PAF)-stimulated platelets. Calyculin A alone caused an increase in protein phosphorylation in unstimulated platelets, with the detection of a number of newly phosphorylated proteins, whereas in PAF-stimulated platelets phosphorylation of the major substrates of protein kinase C and myosin light-chain kinase were no longer transient, but phosphorylation was sustained. PP1/PP2A appear

to play a role in Ca^{2+} homeostasis, as inhibition of PP1/PP2A caused an inhibition of Ca^{2+} mobilization and Ca^{2+} influx through the plasma membrane in PAF-stimulated platelets. The effect of calyculin A on Ca^{2+} mobilization correlated with the observed inhibition of the production of the signal molecule $\text{Ins}(1,4,5)\text{P}_3$. The release reaction (which is a Ca^{2+} -dependent event) was also inhibited by calyculin A. The results are discussed in relation to the possible role of protein kinase C in mediating the events leading to the effects observed with calyculin A.

INTRODUCTION

Agonist–receptor coupling in platelets induces a cascade of biochemical events resulting in a rapid breakdown of inositol phospholipids with the formation of several signal molecules, an elevation of cytosolic Ca^{2+} ($[\text{Ca}^{2+}]_i$) and activation of a number of protein kinases. Indeed, regulation of several functional responses observed upon agonist–receptor coupling have been correlated with protein phosphorylation [1]. Many of the phosphorylation events observed in platelets have been attributed to the role of protein kinase C (PKC) and myosin light-chain (MLC) kinase (MLCK), both of which are serine/threonine-specific protein kinases (for review, see [1]). PKC is a family of isoenzymes which are responsible for the phosphorylation of several target proteins, of which their major substrate in platelets is a protein of 40–47 kDa of unknown function [2]. The PKC isoenzymes possessed by platelets, of which at least five have been demonstrated, are thought to hold a pivotal role in regulating agonist-induced signal transduction [3–5].

Considering, however, that protein phosphorylation is a balance between protein kinase and protein phosphatase activity, relatively little attention has been focused on the role of protein phosphatases in platelet signalling as compared with the extensive investigations carried out to determine the role of protein kinases [6,7]. In particular, little is known about how the phosphorylation events which follow both activation of PKC and other serine/threonine kinases found in platelets are regulated by serine/threonine-specific phosphatases, nor what role these phosphatases have in the signalling pathways of agonist-stimulated platelets. The introduction of both potent and specific inhibitors of serine/threonine-specific phosphatases, e.g. okadaic acid [8], calyculin A [9] and tautomycin [10], is now, however, allowing the role of endogenously activated serine/threonine phosphatases to be further elucidated.

Platelets are known to possess several serine/threonine specific phosphatases, namely type 1 phosphatases (PP1), which are

believed to be responsible for the major portion of serine/threonine phosphatase activity in platelets [11], and type 2 phosphatases (PP2A, PP2B and PP2C) [12–14], although the possibility that other novel types of protein phosphatases may exist should not be dismissed [15]. However, neither the specific role of individual phosphatases nor whether they are responsible for the dephosphorylation of distinct target proteins is known, although it has been suggested that activity of PP1 and possibly PP2A is needed for an agonist to initiate the platelet release reaction [11].

In the present study we have employed the potent and specific serine/threonine phosphatase inhibitor calyculin A, which has been demonstrated to be equipotent against both PP1 and PP2A [9]. Using calyculin A, we have examined the role of serine/threonine phosphatases on the phosphorylation of proteins in unstimulated cells, and also on the regulation of the transient nature of protein phosphorylation in platelet-activating-factor (PAF)-stimulated cells, with particular emphasis on the major 40–47 kDa substrate of PKC, namely pleckstrin. Moreover, the inhibitor has also been used to establish further the role of endogenously activated PP1/PP2A in PAF-induced signal transduction by modulating the function of these phosphatases and investigating the effect of this on PAF-induced changes in signal molecule production ($\text{Ins}(1,4,5)\text{P}_3$ and $[\text{Ca}^{2+}]_i$ elevation) and on the platelet release reaction.

MATERIALS AND METHODS

Materials

Prostacyclin was generously provided by Dr. B. J. R. Whittle, Wellcome Laboratories, Beckenham, Kent, U.K., and used as previously described [16]. 5-Hydroxy[^{14}C]tryptamine (^{14}C]-5-HT) creatinine sulphate (54 mCi/mmol, 50 $\mu\text{Ci}/\text{ml}$), [^{32}P] P_i (5 mCi/ml), $^{45}\text{Ca}^{2+}$ (2 mCi/ml), $\text{Ins}(1,4,5)\text{P}_3$ and [^3H] $\text{Ins}(1,4,5)\text{P}_3$ assay system (code TRK 1000) were purchased from Amersham International. X-ray film was purchased from Fuji. Fura-2

Abbreviations used: PP1, type 1 phosphatases; PP2A, type 2A phosphatases; PKC, protein kinase C; PAF, platelet-activating factor; HBT, Hepes-buffered Tyrode solution; $[\text{Ca}^{2+}]_i$, cytosolic Ca^{2+} concn.; 5-HT, 5-hydroxytryptamine; MLC, myosin light chain; MLCK, MLC kinase; DMSO, dimethyl sulphoxide.

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acetoxymethyl ester (AM) (cell-permeant) was obtained from Molecular Probes (Eugene, OR, U.S.A.), and a stock solution at concentration 5 mM in anhydrous dimethyl sulphoxide (DMSO) was stored at -20°C . PAF (1-*O*-octadecyl-2-acetyl-*sn*-glycero-3-phosphocholine) was supplied by Bachem (Bubendorf, Switzerland). The Ca^{2+} ionophore ionomycin was purchased from Calbiochem and stored at -20°C in DMSO at 5 mg/ml. Calyculin A was purchased from LC Laboratories [distributed by Calbiochem-Novabiochem (UK) Ltd, Nottingham, U.K.]; it was dissolved at 1 mM in DMSO, and further dilutions were made in distilled water. The PKC inhibitor Ro-31 7549/001 was kindly given by Dr. J. S. Nixon, Roche, Welwyn, Herts., U.K.; it was dissolved at 10 mM in DMSO, and further dilutions were made in Hepes-buffered Tyrode solution (HBT; 10 mM Hepes, 145 mM NaCl, 5 mM KCl, 1 mM MgCl_2 , 0.5 mM Na_2HPO_4 , 5.5 mM glucose and 0.25% BSA, pH 7.4).

Preparation of platelets

Washed rabbit platelets were prepared as described for human platelets by Poll et al. [17], by a modification of an original method of Blackwell et al. [18]. The resulting platelet pellet from this preparation was suspended in HBT and the platelet count adjusted. Experiments were carried out at 37°C in the presence of 1 mM external Ca^{2+} .

Protein phosphorylation

Both platelet loading with [^{32}P]P_i and reaction termination were carried out exactly as previously described [16]. [^{32}P]P_i-loaded platelets were incubated with calyculin A alone or before stimulation with PAF as described in the Figure legends. Experiments were carried out both in the absence and presence of unlabelled extracellular phosphate. Proteins (100 μg /track) were separated by SDS/PAGE, and the phosphorylated bands identified by autoradiography were quantified by optical densitometry on a Bio-Rad model GS-670 imaging densitometer.

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. In order to measure the changes in [Ca^{2+}]_i, fluorescence readings were recorded with a PTI dual-wavelength spectrofluorimeter (excitation at 340 nm and 380 nm; emission at 510 nm; 4 nm slit width) fitted with a thermostatically controlled cuvette compartment holder with stirring attachment. The Ca^{2+} -fura-2-fluorescence was calibrated as previously described [19,20]. Mn^{2+} (200 μM) was used in order to determine bivalent-cation influx into cells, and the signal was monitored at 360 nm, a wavelength where the isosbestic point for Ca^{2+} is reached, and therefore one which is insensitive to changes in Ca^{2+} levels but which is quenched by Mn^{2+} [21,22].

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

The [^3H] $\text{Ins}(1,4,5)\text{P}_3$ -specific assay binding kit (RPN 1000) was used to determine extracted $\text{Ins}(1,4,5)\text{P}_3$. $\text{Ins}(1,4,5)\text{P}_3$ production was terminated at 5 s after PAF addition [a time when $\text{Ins}(1,4,5)\text{P}_3$ was significantly elevated above basal [16]] and extracted as previously described [16]. $\text{Ins}(1,4,5)\text{P}_3$ present in the samples was determined by the 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 [23]. 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.

$^{45}\text{Ca}^{2+}$ release from intracellular stores

Platelets were washed in high- K^+ buffer [120 mM KCl, 2 mM KH_2PO_4 , 5 mM $(\text{CH}_3\text{COONa})_2 \cdot 6\text{H}_2\text{O}$, 6 mM MgCl_2 , 20 mM Hepes, in MilliQ water; 5 mM ATP was added, pH adjusted to 6.9 and free Ca^{2+} concentration adjusted below 150 nM] and then suspended to 3×10^9 /ml. The platelets were then permeabilized with 10 μg /ml saponin A, which was removed by further washing in high- K^+ buffer. The intracellular Ca^{2+} stores were loaded with $^{45}\text{Ca}^{2+}$ (2 μCi /ml) for 1 h, and the cells were separated from exogenous $^{45}\text{Ca}^{2+}$ during experimentation on Whatman FP100 filters using a cell harvester. Total release of $^{45}\text{Ca}^{2+}$ from the stores was determined by a 3 min incubation with 75 μM ionomycin. Release of $^{45}\text{Ca}^{2+}$ from the intracellular stores was determined 3 min after the addition of 3 μM $\text{Ins}(1,4,5)\text{P}_3$ by separation of free and retained $^{45}\text{Ca}^{2+}$. $^{45}\text{Ca}^{2+}$ released was determined by liquid-scintillation spectroscopy.

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

Platelets (8×10^8 /ml) were incubated with 200 nCi/ml [^{14}C]5-HT (30 min, 37°C), washed in HBT, and the count was adjusted to 2×10^8 /ml. Percentage [^{14}C]5-HT release was determined as previously described [16].

RESULTS

Inhibition of the activity of PP1 and PP2A on protein phosphorylation in unactivated platelets

Calyculin A (300 nM) induced a time-dependent increase in protein phosphorylation of a number of proteins above their basal levels, reaching a plateau of phosphorylation at 10 min after calyculin A addition (results not shown). Incubation of platelets for 10 min with a range of concentrations of calyculin A (10–300 nM) caused a dose-dependent increase in protein phosphorylation (Figure 1a).

The effect of calyculin A was examined on protein phosphorylation in platelets stimulated with 300 nM PAF (Figures 1b and 1c). Stimulation with PAF alone caused a rapid increase in phosphorylation of the 40 kDa PKC substrate (P40), which reached maximal at 30–60 s after PAF and then declined towards basal levels (Figures 1b and 1d). In order to determine whether the transient nature of phosphorylation of P40 was due to a burst of non-labelled phosphate uptake upon PAF stimulation, as has been described in thrombin-stimulated platelets [24], the experiments were repeated in the absence of extracellular phosphate. Removal of extracellular phosphate had no effect on the transient nature of P40 phosphorylation over the 10 min time course after PAF addition. In contrast, in platelets preincubated for 10 min with 300 nM calyculin A, there was a rapid increase in P40 phosphorylation, but no dephosphorylation (Figures 1c and 1e). In platelets preincubated with calyculin A, phosphorylation of the 22 kDa MLCK substrate (P22) was more rapid, and by 60 s after PAF was 2-fold higher than in the absence of calyculin A (Figures 1b–1e).

Effect of the inhibition of dephosphorylation of proteins by calyculin A on [Ca^{2+}]_i levels

Incubation of platelets for 10 min with calyculin A (10–300 nM) had no significant effect on the basal level of [Ca^{2+}]_i, which was 69.5 ± 7.3 nM. In the presence of 1 mM extracellular Ca^{2+} , activation of platelets with 3 nM PAF caused a transient elevation of [Ca^{2+}]_i, with a maximal elevation of 396 ± 25 nM above basal. Calyculin A pretreatment (10–300 nM) caused a dose-dependent

Figure 1 Effect of inhibition of PP1/PP2A activity on protein phosphorylation in unactivated platelets and in PAF-stimulated platelets

(a) Autoradiograph showing untreated platelets (denoted as lane B) and the effect of increasing concentrations of calyculin A on platelet protein phosphorylation. (b and c) Autoradiographs demonstrating phosphorylation at time points after the addition of PAF in either the absence (b) or presence (c) of 300 nM calyculin A. Corresponding densitometric analyses of the P40 and P22 phosphorylated protein bands indicated in (b) and (c) are given in (d) and (e) respectively. The autoradiographs are each representative of four separate experiments.

inhibition of PAF-induced $[Ca^{2+}]_i$ elevation, with an IC_{50} of 66 ± 4.5 nM (Figures 2a and 2b).

Ca^{2+} mobilization

Extracellular Ca^{2+} was chelated with 2 mM EGTA in order to determine the effect of calyculin A on mobilization of Ca^{2+} from intracellular stores. Replacement of extracellular Ca^{2+} with EGTA decreased the maximum $[Ca^{2+}]_i$ elevation induced by 3 nM PAF (Figures 2c and 2d). Elevation of $[Ca^{2+}]_i$ was inhibited in a dose-dependent manner by treatment with calyculin A, with an IC_{50} of 70.5 ± 11.5 nM.

Bivalent-cation influx

This was determined as described in the Materials and methods section. With this technique, 3 nM PAF caused an increase in the rate of quench of the fluorescence signal, indicating the opening of bivalent-cation channels in the plasma membrane and the influx of bivalent cations (Mn^{2+}) into the cells (Figure 2e). Pretreatment of platelets for 10 min with calyculin A slowed the rate of quench observed upon PAF addition, indicating inhibition of bivalent-cation (Mn^{2+}) movement into the cells (Figure 2e). The concentration range of calyculin A required to inhibit

bivalent-cation influx was similar to that found to inhibit total $[Ca^{2+}]_i$ elevation.

Modulation by PKC

Incubation of platelets for 20 min with the selective PKC inhibitor Ro 31-7549/001 (0.3–10 μ M) caused a dose-dependent inhibition of phosphorylation of the major 40 kDa substrate of PKC (P40) in 300 nM PAF-stimulated platelets (Figure 3a). Ro 31-7549/001 (0.3–10 μ M) alone had no effect on the basal level of $[Ca^{2+}]_i$, nor on the maximal elevation of $[Ca^{2+}]_i$ in PAF-stimulated platelets, although at the highest concentration of Ro 31-7549/001 used (10 μ M) the time taken to reach maximal $[Ca^{2+}]_i$ elevation was slightly slowed (Figure 3c). Figures 3(b) and 3(c) show the effect of Ro 31-7549/001 (10 μ M) on basal $[Ca^{2+}]_i$ levels (Figure 3c) and on PAF-induced $[Ca^{2+}]_i$ elevation (Figures 3b and 3c). Although Ro 31-7549/001 had no effect on the maximal elevation of $[Ca^{2+}]_i$ in PAF-stimulated platelets, it caused a dose-dependent reversal of the inhibitory effect of calyculin A (300 nM) on PAF-induced $[Ca^{2+}]_i$ elevation, with an almost complete reversal of the inhibitory effect at 10 μ M Ro 31-7549/001 (Figures 3b and 3c), a concentration which inhibits PKC-substrate phosphorylation by 95% (Figure 3a). However, the rate at which $[Ca^{2+}]_i$ reached

Figure 2 Effect of calyculin A on PAF-induced $[Ca^{2+}]_i$ elevation

The effect of treatment with calyculin A on 3 nM PAF-induced $[Ca^{2+}]_i$ elevation in the presence of 1 mM $[Ca^{2+}]_i$ (a and b) or 2 mM extracellular EGTA (c and d). (a) and (c) show the fluorescence output from fura-2-loaded platelets after conversion of fluorescence into $[Ca^{2+}]_i$. In (b) and (d) the $[Ca^{2+}]_i$ value represents the peak elevation of cytosolic Ca^{2+} in fura-2-loaded platelets after the basal pre-PAF value has been subtracted. (e) demonstrates the effect of calyculin A on bivalent-cation (Mn^{2+}) influx measured directly as the quenching of the fluorescence signal. The fluorescence traces are from single experiments which are representative of 4–7 experiments, whereas the data in (b) and (d) are means $\pm$ S.E.M. for at least 4 experiments each performed in triplicate.

maximal in non-calyculin A-treated platelets stimulated with PAF was not fully restored by inhibition of PKC (Figure 3c).

Effect of calyculin A on $Ins(1,4,5)P_3$ production and $Ins(1,4,5)P_3$ -induced Ca^{2+} mobilization

Incubation for 10 min with calyculin A (10–300 nM) had

no effect on the basal level of $Ins(1,4,5)P_3$, which was 10.85 ± 4.8 pmol/ 10^9 platelets (Figure 4a). Calyculin A caused a dose-dependent inhibition of PAF-induced $Ins(1,4,5)P_3$ elevation with 300 nM calyculin A preventing elevation of $Ins(1,4,5)P_3$ above the basal level. The IC_{50} value for inhibition of $Ins(1,4,5)P_3$ elevation with calyculin A was 71.3 ± 15.6 nM.

The effect of calyculin A on the binding of $Ins(1,4,5)P_3$ to its

Figure 3 Reversal of calyculin A-induced inhibition of $[Ca^{2+}]_i$ elevation by Ro 31-7549/001 in 3 nM PAF-stimulated platelets

(a) Effect of a 20 min incubation with Ro 31-7549/001 on 300 nM PAF-induced phosphorylation of the major 40 kDa substrate of PKC shown as a percentage (mean $\pm$ S.E.M.) of the absorbance of that for platelets stimulated with 300 nM PAF alone (control). (b) Effect of increasing concentrations of Ro 31-7549/001 (added 20 min before PAF) on 300 nM calyculin A (added 10 min before PAF)-treated platelets stimulated with 3 nM PAF. 'Control' is 3 nM PAF alone, 'Ro + PAF' is 10 μ M Ro 31-7549/001 + 3 nM PAF (no added calyculin); the hatched bars denote the presence of 300 nM calyculin A in platelets pretreated with increasing concentrations of Ro-31-7549 as shown. $[Ca^{2+}]_i$ is the value obtained by subtracting the basal pre-PAF value from the maximal post-PAF value. (c) The corresponding fluorescence output from fura-2-loaded platelets after conversion of fluorescence into $[Ca^{2+}]_i$. Traces: 1, 3 nM PAF; 2, 10 μ M Ro 31-7549 + 3 nM PAF; 3–7 represent treatment with 10, 3, 1, 0.3 or 0 μ M Ro 31-7549, followed by 300 nM calyculin A and then 3 nM PAF. The data are means $\pm$ S.E.M. of 3 separate experiments each performed in triplicate.

receptor was monitored by investigating the release of $^{45}Ca^{2+}$ from intracellular stores of permeabilized platelets with 3 μ M $Ins(1,4,5)P_3$ (Figure 4b). Using this concentration of $Ins(1,4,5)P_3$, $51.6 \pm 3.8\%$ of total $^{45}Ca^{2+}$ was released from the stores, compared with the maximum release of $94.6 \pm 1.2\%$ with 75 μ M ionomycin. Calyculin A had no effect on the ability of $Ins(1,4,5)P_3$ to induce $^{45}Ca^{2+}$ release from the intracellular stores.

Figure 4 Effect of calyculin A on PAF-induced $Ins(1,4,5)P_3$ elevation (a) and $Ins(1,4,5)P_3$ -induced $^{45}Ca^{2+}$ release (b)

Platelets were treated with calyculin A (10–300 nM) for 10 min before activation with 300 nM PAF. $Ins(1,4,5)P_3$ production was terminated at 5 s after PAF (a). Permeabilized platelets were incubated with calyculin A or calyculin A vehicle (V) for 10 min before addition of 3 μ M $Ins(1,4,5)P_3$. Release of $^{45}Ca^{2+}$ was measured at 3 min after $Ins(1,4,5)P_3$ addition (b). The values are means $\pm$ S.E.M. of 3 separate experiments each performed in triplicate.

Figure 5 Effect of calyculin A on PAF- or ionomycin-induced dense-granule release

Platelets were treated for 10 min with calyculin A before stimulation with either 3 nM PAF ($\circ$, $\bullet$) or 5 μ M ionomycin ($\triangle$, $\blacktriangle$). Results are given as a percentage of total possible 5-HT release. The symbols $\circ$ and $\triangle$ (C) denote release of 5-HT by PAF or ionomycin in the absence of calyculin A. The data are means $\pm$ S.E.M. of 3 separate experiments each performed in triplicate.

Effect of calyculin A on PAF- and ionomycin-induced dense-granule release

Platelets release about 60% of 5-HT from their granules in response to stimulation with either 3 nM PAF or 5 μ M ionomycin. At this concentration of ionomycin, which causes sub-maximal dense-granule release, we have previously established that the ionomycin is not causing non-specific cell permeabilization or cell lysis, both by examining the supernatant for lactate dehydrogenase activity (a marker for cell damage) and

also by investigating leakage of low-molecular-mass substances by testing for leakage of fura-2 from the cells [4].

Treatment of platelets with calyculin A caused a dose-dependent inhibition of dense-granule release in platelets stimulated with 3 nM PAF, but had no significant effect on ionomycin-induced dense-granule release (Figure 5). Moreover, as opposed to the inhibitory effect of calyculin A on PAF-induced $[Ca^{2+}]_i$ elevation, treatment with calyculin A was found to have no effect on ionomycin-induced $[Ca^{2+}]_i$ elevation (results not shown).

Effect of calyculin A on $[^3H]$ WEB 2086 binding to platelets

Preincubation of platelets for 10 min with 300 nM calyculin A had no effect on the binding of the PAF antagonist $[^3H]$ WEB 2086 to the PAF receptor on platelets (results not shown).

DISCUSSION

In this study we have used calyculin A, the potent inhibitor of PP1 and PP2A, to investigate PP1/PP2A activity in unstimulated platelets and to elucidate further the role of endogenously activated PP1/PP2A in the stimulus-response coupling pathway(s) of PAF-stimulated rabbit platelets.

Treatment of platelets with calyculin A resulted in a general increase in phosphorylation of all phosphoproteins, similar to the increase in phosphorylation of proteins when PP1/PP2A activity has been inhibited with okadaic acid in a number of different cell types, including hepatocytes (where total ^{32}P labelling was increased 2.5-fold) [8], parotid acini (where only certain proteins appeared to show an increase in phosphorylation) [25], adipocytes [26], neutrophils [27] and platelets [14]. This finding indicates that constitutive protein kinase and phosphatase activities are responsible for the observed pattern of protein phosphorylation and that PP1/PP2A are important in maintaining this basal level of protein phosphorylation. However, despite the increased phosphorylation of proteins in platelets treated with calyculin A, neither the basal level of $Ins(1,4,5)P_3$ nor $[Ca^{2+}]_i$ was altered, suggesting that triggering an increase in these signal molecules requires factors other than simply an increased protein phosphorylation.

In PAF-activated platelets, phosphorylation of the major 40 kDa substrate of PKC was transient, whereas in platelets pretreated with calyculin A maximal phosphorylation was maintained. Tumour promoters such as okadaic acid and calyculin A have no direct effect on the activity of PKC or other protein kinases [9,28], indicating that PP1/PP2A are responsible for the endogenous dephosphorylation of the 40 kDa substrate of PKC. Calyculin A increased the rate at which MLC was phosphorylated in PAF-stimulated platelets, and again the phosphorylation was maintained rather than transient. This finding is interesting, as MLC is the substrate for MLCK, which is a Ca^{2+} - and calmodulin-dependent enzyme that is essentially inactive at $[Ca^{2+}]_i < 1 \mu M$ [29] and, as we have demonstrated in this study, calyculin A inhibits $[Ca^{2+}]_i$ elevation. Calyculin A has no direct effect on MLCK activity [9], but increasing phosphorylation of MLC has been demonstrated to initiate contraction of rat aorta and guinea-pig taenia cecae, due to inhibition of phosphatase activity [30]. MLC is a substrate for PKC in platelets [1], and, as several PKC isoenzymes are Ca^{2+} -independent, it is possible that it is one of these which is causing the observed phosphorylation of MLC at decreased levels of $[Ca^{2+}]_i$; alternatively inhibition of PP1/PP2A may be decreasing the K_a of MLCK for Ca^{2+} and calmodulin [31]. Indeed, a recent study demonstrated that in the presence of okadaic acid the PKC phosphorylation sites of aortic

myosin were phosphorylated and that this phosphorylation was caused by either PKC or another protein kinase [15]. Whatever the identification of the kinase responsible for its phosphorylation, MLC also appears to be a substrate for endogenous PP1/PP2A activity.

The role of endogenously activated PP1/PP2A was also investigated on Ca^{2+} homeostasis in platelets. Calyculin A inhibited both PAF-induced mobilization of Ca^{2+} from the intracellular stores and $Ins(1,4,5)P_3$ production. It is now well established that $Ins(1,4,5)P_3$ is the signal molecule responsible for mobilization of Ca^{2+} from intracellular stores [32,33], and indeed in the present study there is a good correlation between the IC_{50} for the inhibition of $Ins(1,4,5)P_3$ production and that for the inhibition of Ca^{2+} mobilization by calyculin A. Indeed, Lerea [34] demonstrated that calyculin A inhibited phosphatidate formation, which is an indirect method for measuring increase in $sn-1,2$ -diacylglycerol, and which together with the findings in our study on $Ins(1,4,5)P_3$ production suggests that endogenously activated PP1/PP2A have an important role either directly or indirectly at an early stage of the signal pathway in PAF-stimulated platelets. Moreover, the finding that calyculin A has no significant effect on the binding of the PAF antagonist $[^3H]$ WEB 2086 to the PAF receptor indicates that the effects of calyculin A are not due to an antagonistic effect on PAF binding.

In thrombin-stimulated human platelets, however, there is some conflict as to the effect of inhibition of PP1/PP2A on Ca^{2+} mobilization and $Ins(1,4,5)P_3$ production. In a study by Murata and colleagues (1993), calyculin A had no effect on either $Ins(1,4,5)P_3$ production or Ca^{2+} mobilization from intracellular stores in thrombin-stimulated platelets [35]. In contrast, however, a concentration of okadaic acid which was equipotent for PP1 and PP2A inhibited both Ca^{2+} influx and mobilization in thrombin-stimulated platelets [36], and in a separate study okadaic acid was found to inhibit thrombin-induced inositol phosphate production [14].

The same range of concentrations of calyculin A found to inhibit Ca^{2+} mobilization was also found to inhibit the influx of bivalent cations across the plasma membrane in PAF-stimulated platelets. Movement of Ca^{2+} across the plasma membrane may be due to either a second-messenger-operated channel (SMOC) or a receptor-operated Ca^{2+} channel (ROC). If Ca^{2+} influx is solely via a SMOC, the mechanism by which calyculin A inhibited $Ins(1,4,5)P_3$ will also be responsible either directly or indirectly for the inhibition of Ca^{2+} influx through the SMOC. However, if ROCs also exist (for which there is some evidence in platelets for the ADP receptor [37] and the PAF receptor [16,38]), then it would appear that their opening is being regulated by protein phosphorylation and it may be that a dephosphorylation event is required in order to allow opening.

Using p13^{suc1}-Sephacrose, Lerea [34] demonstrated precipitation of a cdc2-related protein kinase (i.e. a cell-cycle dependent protein kinase), which was responsible for calyculin A-sensitive phosphorylation of a 50 kDa protein in unactivated platelets. We have previously demonstrated that, in PAF-stimulated platelets, PKC plays a pivotal and bifurcating role in signal transduction [4,16]. In the present study we have determined the importance of PKC in mediating the effects observed with calyculin A pretreatment in PAF-stimulated platelets by investigating the ability of a selective PKC inhibitor, Ro 31-7549/001, to modify the effect of calyculin A on $[Ca^{2+}]_i$ elevation [5,39]. Treatment of platelets with the PKC inhibitor had no effect either on the basal level of $[Ca^{2+}]_i$ or on maximal $[Ca^{2+}]_i$ elevation in PAF-stimulated platelets. However, Ro 31-7549/001 dose-dependently reversed the inhibitory effect of calyculin A on PAF-induced $[Ca^{2+}]_i$ elevation, although the rate at which $[Ca^{2+}]_i$ elevation reached a

maximum in the absence of calyculin A was not restored. This finding suggests an important role of PKC in mediating the effects of calyculin A in PAF-stimulated platelets. We have previously demonstrated that in PAF-stimulated platelets endogenously activated PKC exerts a negative feedback, decreasing phospholipase C activity [4,16]. The findings from this study suggest that PKC isoenzymes phosphorylate substrates for PP1 and PP2A, and inhibition of dephosphorylation of these substrates potentiates this negative-feedback effect.

Finally, in agreement with studies using thrombin-stimulated human platelets [11,35], inhibition of PP1/PP2A was found to inhibit dense-granule release in PAF-stimulated platelets, indicating either a direct or indirect role for dephosphorylation in dense-granule release. The finding that calyculin A had no effect on $[Ca^{2+}]_i$ elevation or dense-granule release in ionomycin-stimulated platelets, but inhibits both responses in platelets stimulated with PAF, suggests that calyculin A may inhibit PAF-induced granule release (which is a Ca^{2+} -dependent event) indirectly by its inhibition of $[Ca^{2+}]_i$ elevation.

In conclusion, this study has demonstrated that serine/threonine-specific PP1 and PP2A are important in maintaining levels of protein phosphorylation in resting platelets. Inhibition of PP1/PP2A by calyculin A causes an inhibition of signal transduction in PAF-stimulated platelets, preventing increases in the signal molecules $Ins(1,4,5)P_3$ and $[Ca^{2+}]_i$, and also the downstream release reaction, suggesting a positive role for endogenously activated PP1/PP2A in these responses. The findings from this study indicate that the effect of inhibiting phosphatase activity is being exerted either directly or indirectly at an early stage of the signalling pathway, such that calyculin A is either causing an increase in phosphorylation of protein(s) leading to desensitization of the signalling pathway, or is preventing dephosphorylation of constitutively phosphorylated proteins which themselves may have a role in initiating signalling. Finally, from this study it would appear that one of the pivotal regulators of signal transduction, in PAF-stimulated platelets, is the level of phosphorylation of proteins dictated by the balance between PKC and PP1/PP2A activity.

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