The role of Ca^{2+} in regulating the catabolism of PAF-acether (1-*O*-alkyl-2-acetyl-*sn*-glycero-3-phosphocholine) in rabbit platelets

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In the present study we have investigated the effect of changes in the concentration of cytosolic free Ca^{2+} ($[\text{Ca}^{2+}]_i$) on the deacetylation–reacylation of PAF-acether (alkylacetyl-glycerophosphocholine, alkylacetyl-GPC) by rabbit platelets. Washed platelets were incubated with alkyl[^3H]acetyl-GPC (^3H]acetyl-PAF) or ^3H]alkylacetyl-GPC (^3H]alkyl-PAF) and $[\text{Ca}^{2+}]_i$ was subsequently elevated by the addition of the ionophore A23187 or thrombin. The catabolism of PAF-acether was studied by measuring the release of ^3H]acetate or the formation of ^3H]alkylacetyl-GPC. The ionophore inhibited the release of ^3H]acetate and the formation of ^3H]alkylacetyl-GPC with no accumulation of lyso- ^3H]PAF, indicating that the deacetylation of PAF-acether was blocked. The effect of ionophore on the deacetylation of PAF-acether was parallel with the increase of $[\text{Ca}^{2+}]_i$ and could be reversed by the addition of EGTA. In contrast with the prolonged inhibition evoked by ionophore, thrombin, which induced a transient elevation of $[\text{Ca}^{2+}]_i$, merely delayed the deacetylation of PAF-acether. Since intact platelets failed to convert exogenous lyso-PAF, the effect of Ca^{2+} on its acylation was investigated by using platelet homogenates. These experiments showed that the acylation of lyso-PAF was inhibited by the exogenously added Ca^{2+} , with a maximum effect at 1 mM. When the formation of endogenous lyso-PAF from the labelled pool of alkylacetyl-GPC was examined, a prolonged increase in the concentration of lyso-PAF with a parallel and equally prolonged decrease in the cellular level of alkylacetyl-GPC were observed after the addition of ionophore to intact platelets. The addition of EGTA reversed the effect of ionophore, thus permitting reacylation of lyso-PAF. In contrast, only a transient change in the level of lyso-PAF and alkylacetyl-GPC was evoked by the addition of thrombin. Therefore we conclude that the inhibitory effect of Ca^{2+} on the deacetylation–reacylation of PAF-acether may have an important role in the regulation of its biosynthesis.

INTRODUCTION

PAF-acether (platelet-activating factor, 1-*O*-alkyl-2-acetyl-*sn*-glycero-3-phosphocholine, alkylacetyl-GPC), also known as ‘PAF’ is a phospholipid mediator of anaphylaxis and inflammation. It induces various cellular responses, including chemotactic reaction, aggregation and degranulation of platelets and neutrophils. PAF-acether, which is produced by numerous types of cells, is formed by the combined activities of a phospholipase A_2 (PLA_2) and an acetyltransferase [reviewed by Vargaftig *et al.* (1981), Benveniste & Pretolani (1985) and Snyder (1985)]. The conversion (inactivation) of PAF-acether by platelets is catalysed by a sequential deacetylation–reacylation, leading to the formation of alkylacetyl-GPC (Alam *et al.*, 1983; Pieroni & Hanahan, 1983; Touqui *et al.*, 1983, 1985a). The deacetylation step, generating lyso-PAF, is a Ca^{2+} -independent event catalysed by a cytosolic acetylhydrolase (Blank *et al.*, 1981; Kramer *et al.*, 1984; Touqui

et al., 1985b). In alveolar macrophages, lyso-PAF can be reacylated to alkylacetyl-GPC by the combined activities of an acyl-CoA acyltransferase, a CoA-dependent and a CoA-independent transacylase (Robinson *et al.*, 1985). The transacylase-dependent reaction has been shown to be responsible for the transfer of arachidonic acid (AA) from phosphatidylcholine (PC) to lyso-PAF (Kramer *et al.*, 1984; Malone *et al.*, 1985) and is described as the main pathway for the reacylation of lyso-PAF in platelets. It is well documented that the biosynthesis of PAF-acether is a Ca^{2+} -dependent event (Benveniste *et al.*, 1980; Ninio *et al.*, 1982; Gomez-Cambronero *et al.*, 1984, 1985); however, the role of Ca^{2+} in the inactivation of PAF-acether has not been clearly defined. Previous reports are contradictory; for example, in some studies using intact platelets extracellular Ca^{2+} ($[\text{Ca}^{2+}]_o$) deprivation had no effect on PAF-acether catabolism (Alam *et al.*, 1983; Pieroni & Hanahan, 1983; Touqui *et al.*, 1983, 1985a), whereas studies using platelet membranes have shown that acylation of lyso-PAF is inhibited by

Abbreviations used: PAF-acether, platelet-activating factor (1-*O*-alkyl-2-acetyl-*sn*-glycero-3-phosphocholine, alkylacetyl-GPC); alkylacetyl-GPC, 1-*O*-alkyl-2-acetyl-*sn*-glycero-3-phosphocholine; lyso-PAF, lyso-(platelet-activating factor) (alkyl-lysoglycerophosphocholine); PC, phosphatidylcholine; AA, arachidonic acid; TG buffer, Tyrode’s buffer containing 0.25% gelatin; DMSO, dimethyl sulphoxide; CP/CPK, creatine phosphate/creatine phosphokinase; $[\text{Ca}^{2+}]_i$, cytosolic free Ca^{2+} ; $[\text{Ca}^{2+}]_o$, extracellular Ca^{2+} ; ^3H]alkyl-PAF, ^3H]alkylacetyl-GPC; ^3H]acetyl-PAF, alkyl ^3H]acetyl-GPC; PLA_2 , phospholipase A_2 ; BSA, bovine serum albumin; PMA, phorbol myristate acetate; Quin2-AM, Quin2 acetoxymethyl ester; PKC, protein kinase C; IC_{50} , concentration causing half-maximal inhibition.

Ca^{2+} (McKean & Silver, 1985). The discrepancy between these results probably arises from the fact that, in the former studies, the cells were unstimulated: that is, in the absence of stimulation, changes in the concentration of exogenous Ca^{2+} would not significantly alter the Ca^{2+} concentration in the cytosol, where the acetylhydrolase enzyme is located. Both biosynthesis (Ludwig *et al.*, 1985) and catabolism (Pieroni & Hanahan, 1983) of PAF-acether have been shown to be dependent on the concentration of albumin in the extracellular medium. As PAF-acether binds avidly to this protein, it is likely that albumin, by acting as a sink for PAF-acether, effectively reduces the amount of free PAF-acether available for re-uptake, and hence catabolism, by the platelets. Indeed, we have observed that the catabolism of PAF-acether by rabbit platelets was 3–5 times more rapid when the albumin content of the suspension buffer was replaced by gelatin (L. Touqui & B. B. Vargaftig, unpublished work). Thus, during the present study, to assess the effect of Ca^{2+} on PAF-acether catabolism, we chose to replace albumin with gelatin in the suspension medium in order to maximize any effect of Ca^{2+} on PAF-acether catabolism.

MATERIALS AND METHODS

Materials

Bovine serum albumin (BSA; essentially fatty-acid-free), ionophore A23187, phorbol myristate acetate (PMA) and phosphocreatine/creatine phosphokinase (CP/CPK) were obtained from Sigma (St. Louis, MO, U.S.A.). Bovine thrombin was from Hoffman–La Roche (Basel, Switzerland). Ionomycin was from Calbiochem. 1-*O*-[^3H]Octadecyl-2-acetyl-*sn*-glycero-3-phosphocholine ([^3H]alkyl-PAF, 80 Ci/mmol) and 1-*O*-[^3H]octadecyl-*sn*-glycero-3-phosphocholine (lyso-[^3H]PAF; 90 Ci/mmol) were from Amersham International. 1-*O*-Octadecyl-2-[^3H]acetyl-*sn*-glycero-3-phosphocholine ([^3H]acetyl-PAF; 9.8 Ci/mmol) was from CEA (Service des Molecules Marquées, Saclay, France). Synthetic standards (1-*O*-hexadecyl-2-stearoyl-*sn*-glycero-3-phosphocholine, PAF-acether and lyso-PAF) were prepared in the laboratory of Dr. J. J. Godfroid (Université Paris VII, Paris, France). 1-*O*-[^3H]Alkyl-2-acyl-*sn*-glycero-3-phosphocholine was prepared from platelets as described previously (Touqui *et al.*, 1985a). Plastic silica-gel plates for t.l.c., and all solvents, were from Merck.

Methods

Studies with intact platelets. Conversion of PAF-acether into alkylacyl-GPC. Whole blood was collected from the central ear artery of adult New Zealand rabbits into a saline (0.9% NaCl) solution containing 5 mM-EDTA. Formation of platelet-rich plasma and platelet washing procedures have been detailed before (Ardlie *et al.*, 1970). The final platelet suspension, in Tyrode's buffer, pH 7.4, containing MgCl_2 (1 mM), CaCl_2 (2 mM) and gelatin (2.5 mg/ml) (TG buffer), contained 5×10^8 cells/ml. Aliquots (1 ml) of washed platelets were incubated with 0.05 μCi of [^3H]alkyl-PAF (80 Ci/mmol) at 37 °C in the presence and absence of the ionophore A23187 or thrombin under the conditions described in the legend to Fig. 1 (below). After the appropriate incubation times the reactions were terminated by transferring the entire platelet suspension to glass

tubes containing 3 ml of methanol/chloroform (2:1, v/v) at 4 °C. Thereafter the lipids were extracted by the method of Bligh & Dyer (1959), separated by t.l.c. in the presence of authentic standards, and analysed as described previously (Touqui *et al.*, 1985a).

Measurement of [^3H]acetate release from [^3H]acetyl-PAF. Acetylhydrolase activity was measured essentially by the method of Blank *et al.* (1981); briefly: 0.5 ml aliquots of washed platelets were incubated with 0.05 μCi of [^3H]acetyl-PAF (9.8 Ci/mmol), under the conditions detailed in the Figure legends. After predetermined incubation times the reactions were terminated by the addition of 1 ml of chloroform/methanol (1:1, v/v). Phase partition was promoted by centrifugation (2 min at 10000 *g*), and samples from the aqueous phase containing [^3H]acetate were carefully aspirated and their radioactive content measured by liquid-scintillation counting. The efficiency of acetate recovery into the aqueous phase, assessed by using radioactively labelled sodium acetate as an internal standard, was greater than 98%. The recovery of lipids (PAF-acether, alkylacyl-GPC and lyso-PAF) from the organic phase was also assessed by using radioactive standards and was calculated to be greater than 95%.

Studies with endogenous lyso-PAF. In other experiments the endogenous pool of alkylacyl-GPC, the precursor of lyso-PAF, was labelled by the procedures described above. Briefly, platelets (10 ml) were incubated for 2 h with [^3H]alkyl-PAF (0.1 $\mu\text{Ci}/\text{ml}$ final sp. radioactivity), washed twice with Ca^{2+} -free Tyrode's buffer containing 0.25% BSA and resuspended in TG buffer. Aliquots (1 ml) were then stimulated with thrombin (1 unit/ml) or ionophore (1 μM) (see the legend to Fig. 5). The reactions were terminated at the appropriate times and lipids analysed by t.l.c. as described above.

Studies with platelet homogenates. Platelets, prepared as indicated above, were resuspended in Ca^{2+} -free TG buffer (5×10^8 cells/ml) and homogenized as described by Barber & Jamieson (1970). The total homogenate was then resuspended in the same volume of Ca^{2+} -free TG buffer. Aliquots of total homogenates (0.5 ml, containing 50–100 μg of protein) were incubated with lyso-[^3H]PAF (0.05 μCi) in the presence of various concentrations of Ca^{2+} in the medium as shown in Fig. 4 (below). The reactions were then terminated and the lipids extracts analysed as described above.

Measurement of cytosolic free Ca^{2+} concentration ($[\text{Ca}^{2+}]_i$). [$\text{Ca}^{2+}]_i$ was measured by using the Ca^{2+} indicator dye Quin2 as described by Tsien *et al.* (1982). Washed platelets were incubated with 15 μM -Quin2 acetoxymethyl ester (Quin2-AM) (37 °C). After 30 min, loaded platelets were centrifuged in the presence of EDTA (5 mM) (to remove extraneous Quin2-AM) and resuspended in TG buffer (containing $[\text{Ca}^{2+}]_o = 1 \text{ mM}$). Fluorescence changes were monitored in 2 ml platelet samples with a Jobin–Yvon (JY3D) spectrofluorimeter.

RESULTS

The formation of [^3H]alkylacyl-GPC by platelets following the addition of [^3H]alkyl-PAF started after an initial lag time of 30–60 s and reached a maximum conversion of $73 \pm 5\%$ (% of total labelled PAF-acether

Fig. 1. Effect of the Ca²⁺ ionophore A23187 on the conversion of [³H]alkyl-PAF into [³H]alkylacyl-GPC by platelets

Aliquots (1 ml) of washed platelets suspended in TG buffer were incubated simultaneously with 0.05 μ Ci of [³H]alkyl-PAF ([³H]alkylacetyl-GPC) and the ionophore A23187 (1 μ M) in the presence ($\blacktriangle$) or absence ($\bullet$) of 5 mM-EGTA. Control platelets were treated with the ionophore vehicle, 0.2% dimethyl sulphoxide ($\circ$). At the times indicated, the reactions were terminated and lipids analysed by t.l.c. as described in the Materials and methods section. The Figure shows the variation in radioactivity associated with alkylacyl-GPC (—) and PAF-acether (---) as a percentage of the total radioactivity extracted (29074 ± 2661 c.p.m./ml) and represents the results from two separate experiments performed in duplicate. The lyso-PAF content (8–10%), which remained constant over the time course examined, has been omitted from the Figure for clarity. The variation between experiments was $\pm 10\%$.

added) within 30 min. The addition of ionophore A23187 to the platelet suspension (in the presence of [Ca²⁺]_o at 1 mM) immediately preceding the addition of labelled PAF-acether abolished completely the conversion of PAF-acether into alkylacyl-GPC over the time period (0–30 min) examined (Fig. 1). During this period the level of lyso-PAF, which contained 8–10% of the total platelet radioactivity, remained unchanged. The inhibitory effect of the ionophore was not mediated by the release of ADP or by the formation of cyclo-oxygenase metabolites of AA, since pretreatment of the platelets with CP/CPK and/or aspirin did not prevent the ionophore-induced inhibition of alkylacyl-GPC formation (results not shown). However, chelation of extracellular Ca²⁺ by the addition of EGTA (5 mM) prevented the inhibitory effect of the ionophore and, in fact, resulted in a slight increase in the conversion of PAF-acether into alkylacyl-GPC (Fig. 1).

In contrast with the prolonged inhibition produced by the ionophore, the addition of thrombin (1 unit/ml) only increased the lag time of alkylacyl-GPC formation. Indeed, in the presence of thrombin the radioactivity associated with alkylacyl-GPC represented $53 \pm 5\%$ and $96 \pm 3.5\%$ (expressed as a percentage of the correspond-

ing control values; mean $\pm$ S.D. from three experiments) after 10 and 30 min incubation respectively.

As no accumulation of lyso-PAF was observed during the ionophore-induced inhibition of alkylacyl-GPC formation, it would seem likely that deacetylation of PAF-acether was affected. To determine whether this was indeed the case platelets were incubated with [³H]acetyl-PAF and the subsequent deacetylation monitored by measuring the release of [³H]acetate.

Addition of ionophore resulted in a prolonged inhibition of the acetate release (Fig. 2). Here again, the effect of thrombin differs from that of the ionophore in that it only caused an increase in the lag time and displaced the time course of acetate release (the latter reached the control values after 30 min; Fig. 2). Moreover, the inhibition of PAF-acether deacetylation by the ionophore could be reversed by chelating extracellular Ca²⁺ with EGTA, indicating that Ca²⁺ may directly inhibit acetylhydrolase (Fig. 3).

When changes in [Ca²⁺]_i were monitored with Quin2, in the presence of Ca²⁺ at 1 mM, a marked and prolonged rise in [Ca²⁺]_i was observed after the addition of the ionophore ionomycin to platelets. (In the Quin2 studies, ionomycin, which inhibited acetate release in a manner similar to that of A23187, was used as a substitute for A23187, since the latter ionophore fluoresces at the same wavelength as the Quin2 free acid.) We have observed that the addition of EGTA to ionomycin-treated platelets lead to the marked decrease of [Ca²⁺]_i, which returns to the basal levels within 10–15 min (results not shown). In contrast with the prolonged elevation of [Ca²⁺]_i induced by the ionophore, stimulation of the platelets by thrombin produced a transient elevation of [Ca²⁺]_i. Upon stimulation of platelets with either thrombin or ionophore the [Ca²⁺]_i reached a maximal value of 2.0–2.5 μ M. The basal value, in the absence of stimulation, was approx. 80–100 nM. The increase of [Ca²⁺]_i is probably underestimated by this method, since recent studies using aequorin, the Ca²⁺-sensitive photoprotein, showed that [Ca²⁺]_i reached 10-fold higher concentrations during platelet stimulation (Johnson *et al.*, 1985).

In the presence of EGTA (i.e. [Ca²⁺]_o = 0), the elevation of [Ca²⁺]_i induced by the ionophore or thrombin was markedly decreased (results not shown). As shown in Fig. 3, thrombin- and ionophore-induced inhibition of acetate release exhibited a close correlation with the ability of these agents to elevate [Ca²⁺]_i.

On the other hand we examined the possibility that PKC, which is activated by various agonists such as thrombin (Nishizuka, 1984), may influence the catabolism of PAF-acether. To assess this possibility we incubated platelets with PMA (a potent activator of PKC) at concentration of 50 ng/ml, which maximally activates PKC. Under these conditions we observed that the release of acetate was not affected by the addition of PMA over the period (0–30 min) examined, indicating that PKC activation is not involved in the control of acetylhydrolase activity (results not shown).

The conversion of PAF-acether into alkylacyl-GPC is catalysed by the sequential intervention of acetylhydrolase and transacylase, i.e. lyso-PAF is the obligatory intermediate (Touqui *et al.*, 1985a; Robinson & Snyder, 1985). Although the inhibition of acetylhydrolase (first step) would be sufficient to account for the Ca²⁺-induced inhibition of alkylacyl-GPC formation, this does not

Fig. 2. Effect of Ca^{2+} ionophore and thrombin on the release of $[^3\text{H}]$ acetate and on $[\text{Ca}^{2+}]_i$.

Platelets (0.5 ml aliquots) in TG buffer were incubated with 0.05 μCi of $[^3\text{H}]$ acetyl-PAF and 1 μM -ionophore A23187 (open symbols) or 1 unit of thrombin/ml (closed symbols). In separate experiments, aliquots of platelets were loaded with Quin2 and treated with thrombin (1 unit/ml) or ionomycin (0.1 μM). Ionomycin, which completely inhibited acetate release, was substituted for A23187 as the latter ionophore could not be used with the Quin2 system. Changes in $[\text{Ca}^{2+}]_i$ and the release of $[^3\text{H}]$ acetate were measured as detailed in the Materials and methods section. Upon platelet stimulation, $[\text{Ca}^{2+}]_i$ reached a maximal value of 2000–2500 nM (100%), the basal values being estimated at 80–100 nM (0%). The release of acetate reached its maximal value $45\,500 \pm 2500$ c.p.m./ml, $n = 6$) at 30 min. Acetate release from untreated control cells is shown as a broken line and was unaltered by either vehicle. The results are expressed as a percentage of the maximum response observed and are calculated from two or three experiments performed in duplicate.

Fig. 3. Effect of EGTA on the ionophore-induced inhibition of $[^3\text{H}]$ acetate release.

Aliquots of platelets in TG buffer were incubated with $[^3\text{H}]$ acetyl-PAF and A23187 (1 μM) (●) or vehicle (0.2% dimethyl sulphoxide) (○). After 15 min, the aliquots received EGTA (5 mM) (▲) or saline in controls (●). The reactions were terminated, and acetate release measured, as described in the Materials and methods section. Acetate release was calculated as the percentage of maximum and represents the results from two separate experiments performed in duplicate, where the variations were less than 10%.

exclude the inhibition of transacylase (second step) by Ca^{2+} . To resolve this question the effect of Ca^{2+} on the acylation of lyso-PAF was examined. Platelets were homogenized and incubated with lyso- $[^3\text{H}]$ PAF (exogenous lyso-PAF is poorly metabolized by intact platelets; Touqui *et al.*, 1983; Lachachi *et al.*, 1985) in the presence

of various concentrations of Ca^{2+} in the medium. Incubations were conducted in the absence of other cofactors (ATP, CoA, Mg^{2+}). These experimental conditions correspond to those described (Kramer *et al.*, 1984; Malone *et al.*, 1985) for optimal CoA-independent transacylase activity, which appears to be the principal pathway for the acylation of lyso-PAF in platelets. Under these conditions, Ca^{2+} evoked a concentration-dependent inhibition of alkylacyl-GPC formation and a parallel accumulation of lyso-PAF (Fig. 4).

Additional characterization of this enzyme established that it has a pH optimum of 7.5–8.0, was sensitive to *N*-ethylmaleimide ($\text{IC}_{50} = 0.1$ mM) and was unaffected by the addition of CoA, which is in agreement with the characteristics of the CoA-independent transacylase described by Kramer *et al.* (1984).

The deacylation of alkylacyl-GPC by PLA_2 activity is Ca^{2+} -dependent (Albert & Snyder, 1983; Touqui *et al.*, 1985a), thus it could be argued that the apparent inhibition of alkylacyl-GPC formation may be due to Ca^{2+} -induced PLA_2 activation rather than Ca^{2+} -induced transacylase inhibition (i.e. increased catabolism rather than decreased anabolism of alkylacyl-GPC). When the deacylation of alkylacyl-GPC was examined, by incubating purified $[^3\text{H}]$ alkylacyl-GPC with platelet homogenates in the presence of 1 mM- Ca^{2+} , only $25.5 \pm 2.5\%$ (mean $\pm$ S.D. from five separate determinations) of the substrate was hydrolysed. Thus Ca^{2+} -induced PLA_2 activation can only account for part of the apparent inhibition of alkylacyl-GPC formation. Moreover, preincubation of the homogenates with bromophenacyl bromide (0.1 mM), an inhibitor of PLA_2 activity (Benveniste *et al.*, 1980), which prevented $[^3\text{H}]$ AA release under the same conditions, did not abolish the

Fig. 4. Effect of increasing concentrations of Ca²⁺ on [³H]lyso-PAF acylation by platelet homogenates

Platelets were homogenized as described in the Materials and methods section and resuspended in Ca²⁺-free TG buffer. The Ca²⁺ content of duplicate aliquots (0.5 ml, containing 50–100 µg of protein) of total homogenate were then adjusted, by the addition of CaCl₂, to the concentrations indicated on the abscissa. Thereafter each aliquot was incubated with lyso-[³H]PAF (0.05 µCi). After 30 min the reactions were terminated and the radioactivity associated with lyso-PAF (○) and alkylacyl-GPC (●) calculated as a percentage of the total radioactivity extracted (representative of three separate experiments performed in duplicate).

Ca²⁺-induced inhibition of alkylacyl-GPC formation (results not shown).

In addition we have also examined the effect of the ionophore and thrombin on the duration of endogenous lyso-PAF production. The cellular pool of alkylacyl-GPC, the precursor of lyso-PAF, was labelled as described previously (Touqui *et al.*, 1985a), and platelets washed and resuspended in TG buffer. Stimulation of platelets with thrombin produced a transient increase in the concentration of lyso-PAF and, in parallel over the same time course, a transient decrease in the level of alkylacyl-GPC was observed. In contrast, addition of ionophore A23187 produced a prolonged elevation in the concentration of lyso-PAF and an equally prolonged decrease in the level of alkylacyl-GPC, both of which could be reversed by the addition of EGTA (Fig. 5). This suggests that, during platelet stimulation, an increase in the level of [Ca²⁺]_i impairs the acylation of endogenous lyso-PAF, leading to its accumulation within the cell, and confirm the results obtained in the experiments with exogenous lyso-PAF (Fig. 4).

DISCUSSION

Previous reports have shown that the inactivation of PAF-acether by platelets, through a sequential deacetyl-

Fig. 5. Time course of changes in [³H]alkylacyl-GPC and [³H]lyso-PAF content in A23187- and thrombin-treated platelets

Endogenous alkylacyl-GPC was labelled by incubating platelets (10 ml) with [³H]alkyl-PAF (0.1 µCi/ml). After 2 h, extraneous [³H]PAF-acether was removed by washing the cells twice in Ca²⁺-free Tyrode's buffer containing BSA (0.25%). Labelled cells were then resuspended in TG buffer and aliquots (1 ml) were stimulated with 1 µM-ionophore (▲) or thrombin (1 unit/ml) (●). In parallel experiments, EGTA (5 mM) was added to the platelets 15 min after stimulation with ionophore (----). The reactions were terminated at the times indicated on the abscissa and the radioactivity associated with the various lipids assessed as described in the Materials and methods section. The maximum decrease in [³H]alkylacyl-GPC was estimated at 18.4 ± 2.2% and 16.6 ± 3.2% (as compared with the platelet content of [³H]alkylacyl-GPC at zero time) in the presence of ionophore and thrombin respectively; no significant change was observed in vehicle-treated platelets over the time period examined. Results are calculated from two experiments each performed in duplicate and show the variation in radioactivity associated with lyso-PAF (a) and alkylacyl-GPC (b) expressed in c.p.m./ml of platelets. The variation between experiments was ± 10%.

ation-reacylation, is unaffected by changes in the concentration of extracellular Ca²⁺ (Pieroni & Hanahan, 1983; Touqui *et al.*, 1985a). It has therefore been concluded that the inactivation of PAF-acether was a Ca²⁺-independent event; this conclusion must be considered with caution, as the key step in PAF-acether catabolism, namely hydrolysis of the acetyl group (catalysed by acetylhydrolase), occurs within the cytosol. Hence, as the plasma membrane is relatively impermeable to Ca²⁺ in unstimulated platelets, changes in the concentration of [Ca²⁺]_o would not be expected to influence [Ca²⁺]_i.

The present investigation clearly demonstrates that elevating [Ca²⁺]_i with the ionophore A23187 (or ionomycin) evokes a marked and prolonged inhibition of PAF-acether catabolism by platelets. This inhibition is

Scheme 1. Location of the enzymes involved in the conversion of PAF-acether within the cell and their inhibition by the influx of extracellular Ca^{2+}

concomitant with the increase of $[\text{Ca}^{2+}]_i$ and can be reversed by chelation of extracellular Ca^{2+} . In contrast with the prolonged elevation of $[\text{Ca}^{2+}]_i$ produced by the ionophore, thrombin induced a transient elevation of $[\text{Ca}^{2+}]_i$. In the presence of thrombin, the duration of the change of $[\text{Ca}^{2+}]_i$ corresponded to the increased lag time before PAF-acether catabolism commenced. When the effect of Ca^{2+} on PAF-acether catabolism was examined in more detail, we established that both deacetylation of PAF-acether and reacylation of lyso-PAF (at least by a CoA-independent transacylase) were inhibited by Ca^{2+} both *in vivo* and *in vitro*. The physiological relevance of these observations is supported by the effectiveness of extracellular Ca^{2+} in blocking the reacylation of endogenous lyso-PAF produced during A23187-induced platelet activation. Our findings confirm the recent observations of McKean & Silver (1985) in human platelets and support the data reported by Kroner *et al.* (1981) that the incorporation of AA into PC, in bone-marrow-derived macrophages, displayed a marked inhibition in the presence of the Ca^{2+} ionophore A23187. We have previously observed that, in human platelets, thrombin caused a transient formation of PAF-acether the duration of which has been prolonged by a prior incubation of platelets with phenylmethanesulphonyl fluoride, a potent inhibitor of acetylhydrolase (Touqui *et al.*, 1985b). In contrast, no decline was observed in the A23187-induced formation of PAF-acether by rabbit platelets up to 40 min stimulation (Chignard *et al.*, 1980). These findings are in accordance with the results of the present study, i.e. failure of thrombin, in contrast with A23187, to block the catabolism of PAF-acether by rabbit platelets.

The biosynthesis of PAF-acether, by activation of PLA_2 , is known to be a Ca^{2+} -dependent event. The results of the present study indicate that Ca^{2+} potentially inhibits both the deacetylation of PAF-acether and the reacylation of lyso-PAF. An agonist-induced elevation in $[\text{Ca}^{2+}]_i$ is considered to be an important second messenger in platelet reactivity, regulating the various biochemical processes that mediate the cellular response. The physiological relevance of such an event (i.e. elevation of $[\text{Ca}^{2+}]_i$) on PAF-acether metabolism, therefore, would be to promote the accumulation of this endogenous mediator by stimulating its formation and inhibiting its degradation (Scheme 1). In addition, as AA is the major fatty acid incorporated into lyso-PAF, then

inhibition of the reacylation reaction would also be expected to enhance the accumulation of free AA and thus pro-aggregatory icosanoids.

Scheme 1 summarizes the results of the present study and hypothesizes a proposal for the Ca^{2+} -mediated regulation of PAF-acether catabolism within platelets.

We thank Dr. A. Vanhove (CEA, Saclay, France) for the preparation of $[\text{^3H}]$ acetyl-PAF used in this study.

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