Evidence that cytosolic phospholipase A_2 is down-regulated by protein kinase C in intact human platelets stimulated with fluoroaluminate

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Abstract We reported that protein kinase C (PKC) inhibitors increase the release of arachidonic acid induced by fluoroaluminate (AlF_4^-), an unspecific G-protein activator, in intact human platelets. Now we demonstrate that this effect is independent of the extracellular Ca^{2+} concentration and that AlF_4^--induced release of AA is abolished by BAPTA, an intracellular Ca^{2+} chelator, even in the presence of GF 109203X, a specific and potent PKC inhibitor. This compound also blocks the liberation of the secretory phospholipase A_2 in the extracellular medium, indicating that this enzyme is not involved in the potentiation of arachidonic acid by PKC inhibitors. On the other hand, the latter effect is completely abolished by treatment of platelets with $AACOCF_3$, a specific inhibitor of cytosolic phospholipase A_2 ($cPLA_2$). These observations indicate that $cPLA_2$ is responsible for the AlF_4^--induced release of arachidonic acid by a mechanism that is down-regulated by PKC.

Key words: Phospholipase A_2 activation; Human platelet; Protein kinase C inhibitors; Secretory phospholipase A_2; Cytosolic phospholipase A_2; G-protein

1. Introduction

Agonist-activated platelets produce free fatty acids from the hydrolysis of membrane phospholipids through the activation of lipolytic enzymes that are almost inactive in the resting state. Particular attention has been devoted to the mechanisms controlling the levels of free arachidonic acid (AA) because it is the substrate for enzymes leading to the production of powerful lipid mediators. Nowadays, there is a general consensus that the activation of the Ca^{2+}-dependent cytosolic phospholipase A_2 ($cPLA_2$) is required for receptor-mediated liberation of AA in several cell types including human platelets [1]. The activation of this enzyme requires Ca^{2+}-mediated translocation and phosphorylation [1–3] but, while the requirement of an increase of cytosolic Ca^{2+} concentration is considered to be essential for the activation of $cPLA_2$, it is still unclear whether its phosphorylation is strictly required [3]. In addition, the identity of the kinase(s) involved in receptor-mediated phosphorylation of $cPLA_2$ in intact cells is not completely defined.

Previous studies have demonstrated that protein kinase C (PKC) activators have a synergistic effect on the production of free AA induced by Ca^{2+} ionophore that is abolished by PKC inhibitors [4,5].

Our laboratory has recently reported that PKC inhibitors do not affect the release of AA in thrombin-stimulated human platelets but, surprisingly, they increase that induced by the stimulation with fluoroaluminate (AlF_4^-), an unspecific and widely used G-protein activator [6]. This finding suggests that, in intact human platelets, G-protein-induced liberation of arachidonic acid may take place by an unknown mechanism that is down-regulated by PKC. Opposite effects were reported by Akiba et al. [7] with rabbit permeabilized platelets, where the liberation of AA induced by AlF_4^- was potentiated by phorbol 12-myristate 13-acetate (PMA) and inhibited by staurosporine in the presence of 100 μM external Ca^{2+}. The aim of the present study was the investigation of the nature of the PLA_2 potentiated by PKC inhibitors in intact human platelets stimulated by AlF_4^-.

To this end we assessed: (i) the Ca^{2+} dependence of AA liberation; (ii) the role of the released secretory PLA_2 (sPLA2); (iii) the effect of a selective $cPLA_2$ inhibitor.

Our results demonstrate that AlF_4^- triggers $cPLA_2$ activation by a mechanism that does not depend on the extracellular Ca^{2+} concentration and is up-regulated by the inhibition of PKC.

2. Materials and methods

2.1. Materials

[5,6,8,9,11,12,14,15-^{3}H]Arachidonic acid ([^{3}H]AA; 191–220 Ci/mmol) was purchased from NEN Research Products (Boston, MA, USA). [9,10(N)- ^{3}H]Oleic acid was from Amersham Pharmacia Biotech (Essex, UK). Prostacyclin (PGI_2), bovine serum albumin (BSA fraction V) and apyrase were from Sigma Chemical (St. Louis, MO, USA). Bovine thrombin (Topostasin) was from Roche (Basel, Switzerland). Fura 2-AM, BAPTA-AM, staurosporine, H-7 (1-(5-isoquinolinylsulfonyl)-2-methylpiperazine), $AACOCF_3$ (arachidonyltrifluoromethyl ketone), bisindolylmaleimide I (GF-109203X) and bisindolylmaleimide V were purchased from Calbiochem-Novabiochem Corporation (La Jolla, CA, USA). Silica gel 60A plates (Whatman International Ltd, Maidstone, UK) were used for thin-layer chromatography.

2.2. Preparation of human platelets

Blood was obtained from healthy volunteers using ACD as anticoagulant. Platelets were pelleted in the presence of 0.2 μM PGI_2 and

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Abbreviations: AA, arachidonic acid; $AACOCF_3$, arachidonyltrifluoromethyl ketone; H-7, 1-(5-isoquinolinylsulfonyl)-2-methylpiperazine; GF 109203X, bisindolylmaleimide I; MAPK, mitogen-activated protein kinase; PGI_2, prostacyclin; PKC, protein kinase C; PLC, phospholipase C; PMA, phorbol 12-myristate 13-acetate; $cPLA_2$, cytosolic phospholipase A_2; $sPLA_2$, secretory phospholipase A_2; Thr, thrombin; WLP, washed labelled platelets

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50 U/ml heparin and then resuspended in Tyrode's buffer containing 0.35% BSA, apyrase (20 μg/ml) and PGI_2 (0.2 μM). This procedure was repeated twice to obtain washed platelets (WP) which were finally resuspended, unless differently indicated, in Tyrode's buffer containing 0.35% BSA without addition of Ca^{2+} and Mg^{2+} (standard Tyrode's buffer) [6].

2.3. Preparation of platelets loaded with Fura 2-AM

WP were resuspended in standard Tyrode's buffer and the volume was adjusted to give 5×10^8 cells/ml and then incubated with 4 μM Fura 2-AM at 37°C for 45 min [8]. Platelets were then pelleted and resuspended in Tyrode's buffer (2.5×10^8 cells/ml). When indicated, platelets were incubated with Fura 2-AM and 100 μM BAPTA/AM or with a corresponding volume of its vehicle (DMSO).

2.4. Stimulation of platelets and determination of cytosolic free Ca^{2+}

Fura 2-loaded platelet suspensions (0.5 ml) were placed in a cuvette, kept at 37°C under continuous stirring, in the sample holder of a RF-5000 spectrofluorimeter (Shimatzu Co., Kyoto, Japan) equipped with software for the calculation of Ca^{2+} concentration, according to Grynkiewicz et al. [9] using a dissociation constant for Fura 2 of 224 nM. This task was achieved by monitoring the fluorescence ratio at two excitation wavelengths (336 and 380 nm) with emission set at 510 nm. R_{max} was determined by lysing platelets with 0.2% Triton X-100 in the presence of a saturating Ca^{2+} concentration. R_{min} was determined by addition of 10 mM EGTA. Ca^{2+} concentration in the standard Tyrode's buffer was determined as above using Fura 2 instead Fura 2-AM.

2.5. Preparation of platelets labelled with [^{3}H]arachidonic acid

Washed labelled platelets (WLP) were prepared as previously reported [6]. Briefly, resuspended platelets were incubated with [^{3}H]AA (1 μCi/ml of platelet suspension) at 37°C for 1 h. Further washes removed the excess of labelled precursor. Finally, WLP were resuspended (2.5×10^8/ml) in a convenient volume of standard Tyrode's buffer. When indicated platelets were loaded with BAPTA/AM (100 μM for 45 min at 37°C) [8].

2.6. Stimulation of WLP and determination of the radioactivity associated with arachidonic acid

WLP suspensions (0.5 ml) were stimulated with thrombin 0.5 U/ml for 3 min or with AlF_4^- (NaF and $AlCl_3$ solution in a 5000:1 molar ratio) for 20 min under continuous stirring at 1100 rpm. When indicated, WLP were preincubated with PMA (100 nM for 5 min), staurosporine (200 nM for 4 min), H-7 (200 μM for 4 min), GF 109203X (11 μM for 5 min) or $AACOCF_3$ (20–60 μM for 2 min) [10] before stimulation with AlF_4^- or with thrombin. Platelet aggregation was monitored by photometry (Aggrecorder, Menarini, Milan, Italy). Lipid extraction, AA isolation and determination of radioactivity were performed as previously described [6].

2.7. Assay of platelet $sPLA_2$

Platelet $sPLA_2$ shows a marked preference for substrate presented as *Escherichia coli* membranes [11] which contain mainly phosphatidylethanolamine (≈70% of total phospholipids). Thus, [^{3}H]oleic acid-labelled *E. coli* is generally used as a substrate for assaying non-pancreatic $sPLA_2$. This substrate was prepared according to Patriarca et al. [12] and washed as described by Kramer and Pepinski [13]. The suitability of this substrate was checked using bee venom PLA_2. After stimulation, platelets (5×10^8/ml) were pelleted and convenient aliquots of supernatants were incubated with autoclaved [^{3}H]oleic acid-labelled *E. coli* (approximately 30 nCi/sample). Enzyme assay was carried out according to Mueller et al. [14] in a total volume of 0.3 ml in the presence of 5 mM $CaCl_2$ and 1 mg/ml BSA.

2.8. Statistical analysis

Data were analyzed by ANOVA followed by multiple comparison (Scheffé's test).

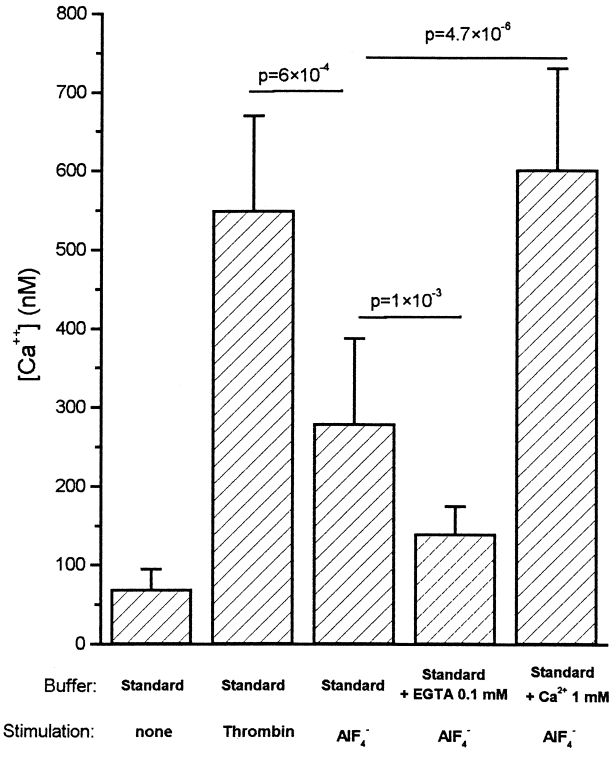

Fig. 1. Effect of extracellular Ca^{2+} concentration on free cytosolic Ca^{2+} concentration in stimulated intact human platelets. WP were loaded with Fura 2-AM and stimulated with AlF_4^- (21 mM NaF for 20 min) or thrombin (0.5 U/ml for 3 min). Data are expressed as nM cytosolic Ca^{2+} and are the mean ± S.D. (n = 7–15).

Table 1
Effect of extracellular Ca^{2+} concentration and PKC inhibitors on free cytosolic Ca^{2+} changes and on AA release induced by AlF_4^- in human intact platelets

Addition	Standard buffer+EGTA (0.1 mM)		Standard buffer		Standard buffer+Ca^{2+} (1 mM)	
	$[Ca^{2+}]_{stim}/[Ca^{2+}]_{rest}$	AA (nCi/10^9 platelets)	$[Ca^{2+}]_{stim}/[Ca^{2+}]_{rest}$	AA (nCi/10^9 platelets)	$[Ca^{2+}]_{stim}/[Ca^{2+}]_{rest}$	AA (nCi/10^9 platelets)
None	1	13.9 ± 3.7	1	10.6 ± 3.0	1	13.7 ± 4.7
AlF_4^-	3.9 ± 2.7	47.3 ± 7.4*	4.2 ± 1.1	49.6 ± 13.0*	5.8 ± 2.3	49.3 ± 14.2*
+Staurosporine	3.6 ± 0.6	120.4 ± 36.6**	3.7 ± 0.9	103.3 ± 30.0**	4.2 ± 1.5	121.0 ± 37.5**
+H-7	2.5 ± 0.2	125.3 ± 40.8**	2.6 ± 0.4	125.4 ± 36.4**	4.5 ± 0.1	143.3 ± 41.0**
+GF-109203X	4.5 ± 1.5	128.4 ± 16.0**	3.2 ± 0.5	123.1 ± 25.0**	6.4 ± 2.6	160.4 ± 31.3**

Platelets were treated with staurosporine (200 nM for 4 min), H-7 (200 μM for 4 min) or GF 109203X (11 μM for 5 min) before stimulation with AlF_4^- (21 mM for 20 min). Calcium data are expressed as the ratio between Ca^{2+} concentration measured immediately before stimulation ($[Ca^{2+}]_{rest}$) and the maximal concentration attained after addition of AlF_4^- ($[Ca^{2+}]_{stim}$). All data are the mean ± S.D. from at least two experiments in duplicate.
*$P < 0.05$ versus none.
**$P < 0.05$ versus AlF_4^-.

3. Results

3.1. Effect of extracellular Ca^{2+} concentration on AlF_4^--induced cytosolic free Ca^{2+} concentration and arachidonic acid release

The concentration of cytosolic free Ca^{2+} in unstimulated human platelets, resuspended in Tyrode's buffer without Mg^{2+} and Ca^{2+} added (standard Tyrode's buffer), was 68 ± 26 nM ($n = 15$). The concentration of Ca^{2+} in this buffer was 2 μM.

As shown in Fig. 1, after the stimulation of platelets with 0.5 U/ml thrombin in standard buffer, the maximal concentration of cytosolic free Ca^{2+} was 551 ± 172 nM ($n = 10$) in accordance with previous reports [15,16]. Under the same conditions AlF_4^- (21 mM NaF) increased free Ca^{2+} to 280 ± 16 nM ($n = 15$). Preincubation with aspirin (200 μM for 30 min) did not affect the Ca^{2+} rise induced by AlF_4^-. In a nominally Ca^{2+}-free medium (0.1 mM EGTA added 2 min before stimulation) Ca^{2+} level in resting platelets was 31.9 ± 11.0 and the addition of AlF_4^- brought intracellular

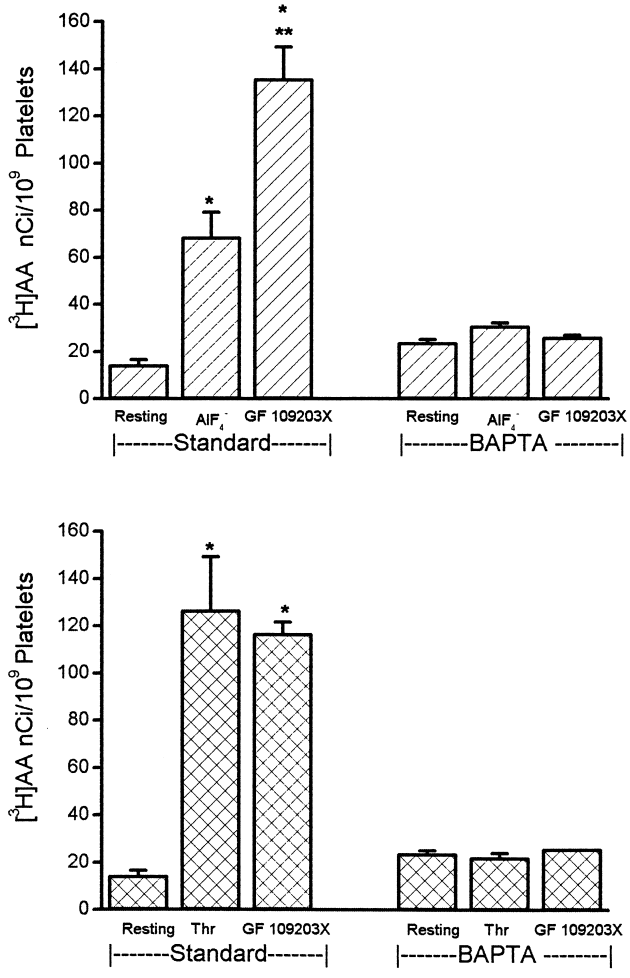

Fig. 2. Effect of intracellular Ca^{2+} chelation on arachidonic acid release. WLP were loaded with BAPTA/AM (100 μM) and then stimulated with AlF_4^- (21 mM NaF) for 20 min or thrombin (0.5 U/ml for 3 min). When indicated WLP were preincubated with GF 109203X (11 μM for 5 min). Data are the mean ± S.D. from two experiments in duplicate. $^*P < 0.05$ versus resting; $^{**}P < 0.05$ versus AlF_4^-

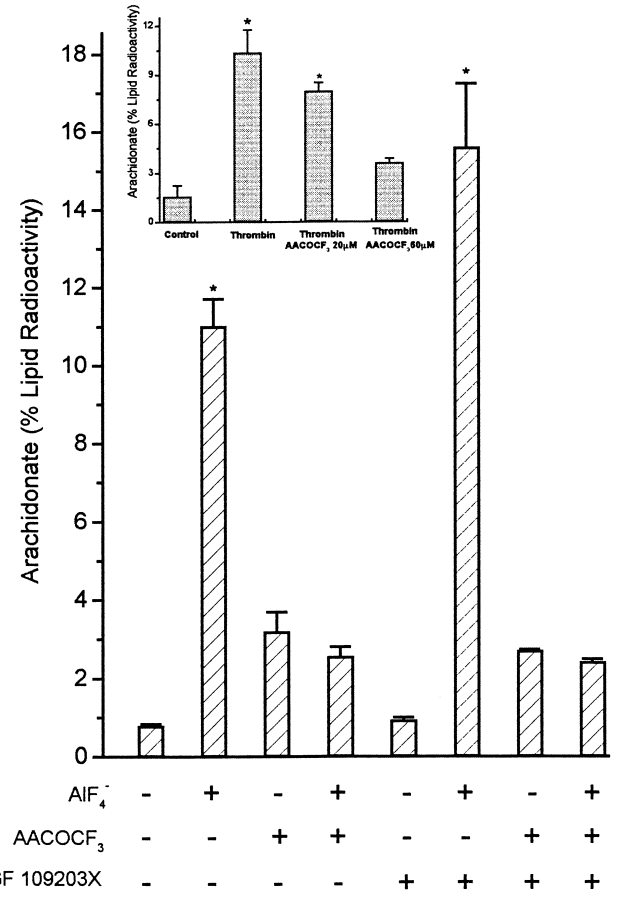

Fig. 3. Effect of arachidonyltrifluoromethyl ketone on the release of arachidonic acid induced by AlF_4^-. WLP were resuspended in Tyrode's buffer containing 0.05% BSA, preincubated with $AACOCF_3$ (20–60 μM for 2 min) and/or GF 109203X (11 μM for 5 min) and stimulated with AlF_4^- (21 mM NaF) for 20 min. Data are expressed as radioactivity associated with AA (% of total lipid radioactivity) and are the mean ± S.D. of two experiments in duplicate. $^*P < 0.05$ versus control.

Ca^{2+} to 138 ± 37 nM ($n = 7$). On the other hand, when platelets were exposed to 1 mM Ca^{2+} for 2 min, the stimulation with AlF_4^- caused an increase in cytosolic free Ca^{2+} concentration that reached a value of 602 ± 129 nM ($n = 7$). These observations confirm previous results [15] indicating that the increase of cytosolic free Ca^{2+} produced by AlF_4^- is due to both Ca^{2+} influx and mobilization from intracellular stores.

PKC inhibitors (staurosporine, H-7 or GF-109203X) did not cause any significant change in the levels of fluorescence with respect to control samples up to 25 min after their addition to the media and they did not significantly interfere with the rise of cytosolic Ca^{2+} induced by AlF_4^-, either in the absence or in the presence of extracellular Ca^{2+} (Table 1). Similarly, non-significant changes were observed after preincubation of platelets with PMA (100 nM for 5 min).

When WLP were stimulated with AlF_4^- (21 mM NaF) under identical conditions the production of [^{3}H]AA was absolutely independent of the extracellular Ca^{2+} concentration. However, staurosporine, H-7 or GF 109203X caused a large increase in [^{3}H]AA after stimulation with AlF_4^- which was also independent of the extracellular Ca^{2+} concentration (Table 1), while an analogue of GF 109203X (bisindolylmaleimide V) inactive on PKC did not affect AlF_4^--induced AA

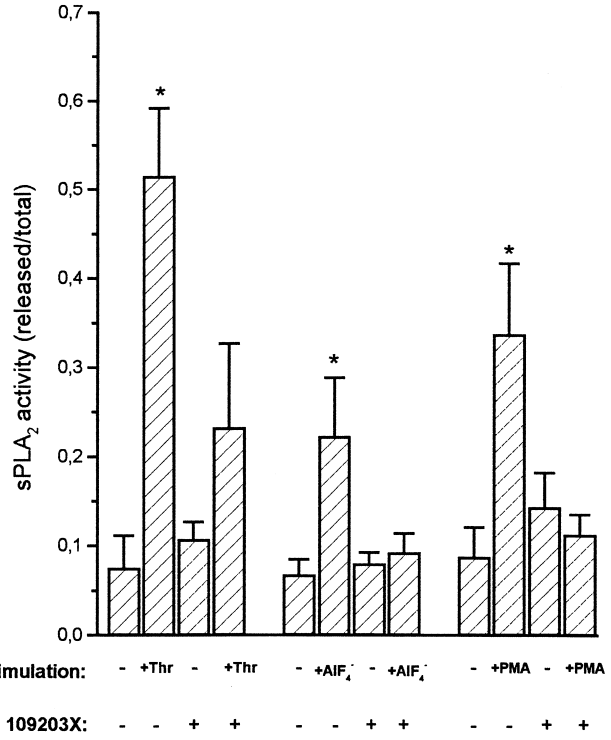

Fig. 4. Effect of PKC inhibition on the release of $sPLA_2$. WP were resuspended in Tyrode's buffer and, after preincubation with GF 109203X (11 μM for 5 min), were stimulated with AlF_4^- (21 mM NaF for 20 min) or Thrombin (0.5 U/ml for 3 min) or PMA (100 nM for 5 min). After stimulation platelets were pelleted and $sPLA_2$ activity was assayed in the supernatants and in platelet lysates. Data (mean ± S.D.) are from two experiments in duplicate and are expressed as a fraction of activity released with respect to total. $*P < 0.05$ versus control.

release (data not shown). The potentiation of AA release due to PKC inhibitors was also observed in aspirin-treated platelets (data not shown). In some experiments, platelets were resuspended in standard Tyrode's buffer, preincubated with 100 μM BAPTA/AM [8] and then loaded with Fura-2. The stimulation of BAPTA-treated platelets with AlF_4^- caused a slight but significant increase in cytosolic free Ca^{2+} that, however, always remained below the levels observed in resting platelets kept in standard buffer (data not shown).

The chelation of intracellular Ca^{2+} with BAPTA completely blocked the release of labelled AA in both thrombin- and AlF_4^--stimulated platelets, even when preincubated with GF 109203X (Fig. 2).

3.2. $cPLA_2$ inhibition and release of arachidonic acid

$AACOCF_3$ (60 μM), a specific $cPLA_2$ inhibitor, blocked the liberation of AA induced by AlF_4^- even in the presence of GF 109203X (Fig. 3) and it caused a dose-dependent reduction of the release of labelled AA in thrombin-stimulated platelets (Fig. 3, inset). It should be mentioned that $AACOCF_3$ (20–60 μM) caused a dose-dependent increase in the radioactivity associated with AA in unstimulated platelets, in agreement with previous reports [17] (data not shown).

3.3. Release of $sPLA_2$

$sPLA_2$ is released in the extracellular medium following stimulation of human platelets with PMA, thrombin or AlF_4^-. The preincubation with GF 109203X inhibited the release of the enzyme in the extracellular medium induced by these agonists (Fig. 4).

4. Discussion

AlF_4^- is a widely used tool for testing the involvement of trimeric G-proteins in a given pathway because it binds to G_α-GDP mimicking the γ-phosphate of GTP [18]. The exposure of intact platelets to AlF_4^- induces aggregation, release of granule contents and liberation of AA from membrane phospholipids [19]. The latter event is achieved by a mechanism, as yet uncharacterized, which is distinct from that utilized by thrombin. In fact, the treatment of intact human platelets with neomycin or sodium nitroprusside inhibits the liberation of AA induced by thrombin but not by AlF_4^- [19]. On the other hand, inhibitors of PKC potentiate the release of AA and do not affect that induced by thrombin [6]. Our hypothesis is that AlF_4^- triggers a G-protein-mediated mechanism that leads to the liberation of AA, which is specifically inhibited by one or more phosphorylation reactions catalyzed by PKC. The identity of the phospholipase activated by AlF_4^- and regulated by PKC is unknown so far.

Halenda et al. [4] reported that PKC activators potentiate the release of AA induced by Ca^{2+} ionophores in human platelets, an effect suppressed by PKC inhibitors. Similar results were obtained with permeabilized rabbit platelets in the presence of 1 mM Ca^{2+} or stimulated with GTP-γ-S or AlF_4^- in the presence of 100 μM Ca^{2+} [7]. These observations are clearly in contrast with our results obtained with intact platelets stimulated with AlF_4^- in the presence of 2 μM external Ca^{2+}[6]. However, it should be considered that the treatment with Ca^{2+} ionophore or the permeabilization of platelets, in the presence of relatively large Ca^{2+} concentrations, may cause an increase in cytosolic concentration much larger than that attainable in intact platelets stimulated under physiological conditions. In this study we have shown that AlF_4^- stimulation causes an increase in cytosolic Ca^{2+} that varies depending on the extracellular concentration of the cation but it never exceeds that induced by thrombin [16]. Furthermore, we have demonstrated that the rise of cytosolic Ca^{2+} concentration induced by AlF_4^- is not affected by PKC inhibitors. This excludes the possibility that the large increase in AA release provoked by PKC inhibitors and observed during stimulation of intact platelets with AlF_4^- is related to an influence on the cytosolic Ca^{2+} concentration.

We have also demonstrated that AlF_4^- induces the release in the extracellular medium of $sPLA_2$, a finding not previously reported, showing that the release of this enzyme is a G-protein-mediated process. PKC inhibitors abolished the release of $sPLA_2$, thus a contribution of this enzyme to the release of AA induced by AlF_4^- can be excluded. This conclusion is further supported by the observation that the potentiation of the AlF_4^--induced release of AA by PKC inhibitors is also observed with EGTA in the medium, since $sPLA_2$ requires millimolar concentrations of Ca^{2+} for optimal activity [14].

The observation that the liberation of AA induced by AlF_4^- is strictly Ca^{2+}-dependent and takes place with submicromolar concentrations of cytosolic Ca^{2+} suggests that its potentiation by PKC inhibitors is somehow related to the activation of $cPLA_2$. This assumption is further supported by the observa-

tion that a selective inhibitor of this enzyme suppresses the AlF_4^--induced release of AA and the effect of PKC inhibitors.

$cPLA_2$ has various sites for potential phosphorylation by serine/threonine and tyrosine kinases and consensus sequences for mitogen-activated protein kinase (MAPK) as well for PKC and PKA [20]. However, the nature of the protein kinase(s) involved in the phosphorylation of platelet $cPLA_2$, induced by physiological agonists or non-physiological compounds, is still unclear. Particularly, collagen and thrombin activate $cPLA_2$ by a PKC-independent mechanism [21] and $p38^{mapk}$ appears to be involved in this process [22,23]. On the other hand, PKC-mediated phosphorylation of $cPLA_2$ by $p42/p44^{mapk}$ takes place in platelets treated with phorbol esters and Ca^{2+} ionophore [24].

These observations and our results suggest that, in AlF_4^--stimulated platelets, PKC has to exert a down-regulation of the liberation of AA upstream of the phosphorylation and activation of $cPLA_2$. The site of this effect is still unknown but it is reasonable to suppose that it does not belong either to the pathways activated by collagen or thrombin, since those are not or little affected by specific PKC inhibitors [6,22], or to that triggered by phorbol esters and Ca^{2+} because it is blocked by PKC inhibitors.

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