Protein kinase C inhibitors enhance G-protein induced phospholipase A2 activation in intact human platelets

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Abstract Washed intact human platelets were prelabelled with [3H]arachidonic acid ([3H]AA) and stimulated with thrombin or with AIF4, a known unspecific activator of G-proteins. Both stimuli induced the liberation of [3H]AA, the release of β-thromboglobulin (β-TG) and platelet aggregation. PMA did not induce liberation of [3H]AA although it induced β-TG release and aggregation; preincubation with PMA did not modify significantly the amounts of [3H]AA and β-TG released by thrombin or AIF4. Different inhibitors of PKC (staurosporine, H-7 and calphostin C) increased the release of [3H]AA and inhibited β-TG release and aggregation induced by AIF4 but they had no effect when platelets were stimulated with thrombin (0.5 U/ml). Calphostin C was able to release [3H]AA by itself without inducing aggregation or β-TG release. Okadaic acid (a serine/threonine phosphoprotein phosphatase inhibitor) greatly inhibited the release of [3H]AA, β-TG and aggregation in AIF4-stimulated platelets. These results indicate the presence of a G-protein mediated mechanism for the activation of a platelet phospholipase A2 which is negatively affected by a protein kinase, sensible to putative inhibitors of protein kinase C, and it is activated by a protein phosphatase, sensible to okadaic acid.

Key words: Phospholipase A2 activation (human platelets); Protein kinase C inhibitors; G-Protein; β-Thromboglobulin release; Protein phosphatase

1. Introduction

Type II phospholipase A2 (sPLA2) and type IV phospholipase A2 (cPLA2) have been detected and characterized in human platelets. The former enzyme is released from stimulated platelets and it is identical to the PLA2 present in rheumatoid synovial fluid [1,2]. Thus, this enzyme is supposed to play a role in extracellular fluids after its release from secretory granules where it is stored [3]. Whether sPLA2 contributes to the liberation of arachidonic acid (AA) in stimulated platelets is still matter of debate. On the other hand, the involvement of cPLA2 in the receptor-coupled release of AA has been well documented in platelets and in other cell types [4-6]. This enzyme has a cytosolic localization, it is highly specific for arachidonoyl residues [4] and it requires submicromolar concentration of Ca2+ and phosphorylation for full activation [5]. Since the activation of cPLA2 is of primary importance for the generation of bioactive lipid messengers, the elucidation of the mechanisms that regulate the activity of this enzyme is a key issue for understanding some aspects of signal transduction in platelets.

We have previously compared the effects of an unspecific and direct activator of G-proteins, fluoroaluminate (AIF4), with those of thrombin on PLA2 activation as well as aggregation and release of granule contents in intact human platelets. We have demonstrated that PLA2 activation and the release of β-TG can be induced directly by a G-protein dependent mechanism, which does not require a previous activation of phospholipase C (PLC) [7], differently from what happens when thrombin is the stimulus.

The stimulation of human platelets with thrombin activates various protein kinases [8] and cPLA2 is supposed to be the substrate of one of them, at least. Mitogen-activated protein kinases (MAPK) have been proposed as the enzymes involved in cPLA2 phosphorylation following the activation of MAPK itself by a tyrosine kinase in thrombin-stimulated platelets [9]. However, the involvement of proline-directed kinases, other than MAPK, in the activation of platelet cPLA2 cannot be ruled out [10].

In other cell types, PKC-dependent and PKC-independent mechanisms for the activation of MAPK and consequently of cPLA2 have been proposed [11].

In platelets, the role of PKC in G-protein mediated activation of PLA2 is not well defined. In saponine permeabilized rabbit platelets, phorbol 12-myristate 13-acetate (PMA) potentiates the release of AA induced by non-hydrolysable analogs of GTP or by Ca2+ addition [12]. In intact human platelets, PMA potentiates Ca2+-ionophore induced AA release [13] whereas it inhibits the formation of thromboxane B2 (TXB2) caused by AIF4 addition [14].

AIF4 induces platelet activation by mechanisms not fully elucidated. This compound (10 mM or less) produces diacylglycerol and TXB2 without activating PLC, and increases PKC activity without an apparent increase of intracellular Ca2+ [15]. At higher concentrations (20 mM or more), AIF4 activates PLC and liberates TXB2 being the two events independent [7,14].

In order to focus upon the role of PKC on the release of AA by a G-protein mediated activation of PLA2, we have assessed the effects of PMA (PKC activator) or known inhibitors of protein kinases (staurosporine, H-7 and calphostin C) or of protein phosphatases (okadaic acid) on the production of radioactive AA in human intact platelets, prelabelled...
with $[^{3}H]AA$, and stimulated with AlF$_4^-$, a direct activator of G-protein. The results of these experiments have been compared to those obtained by stimulating platelets with thrombin through a receptor-mediated mechanism. In both cases, functional parameters as platelet aggregation and secretion have been also determined.

2. Materials and methods

2.1. Materials

[5,6,8,9,11,12,14,15]Arachidonic acid ($[^{3}H]AA$; 191-220 Ci/ mmol) was purchased from NEN Research Products (Boston, MA, USA). Prostacyclin (PGI$_2$), bovine serum albumin (BSA fraction V), and apyrase were from Sigma Chemicals (USA). Bovine thrombin (Topostasin) was from Roche (Basel, Switzerland). Staurosporine, H-7 (1-(5-isoquinolinylsulfonyl)-2-methylpiperazine), calphostin C and okadaic acid were purchased from Calbiochem-Novabiochem Int. (San Diego, CA, USA). Silica gel 60A plates (Whatman Int. Ltd., Maidstone, UK) were used for thin-layer chromatography.

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

Washed labelled platelets (WLP) were prepared as previously reported [7]. Briefly, blood (50-80 ml) was drawn from healthy volunteers in citrate-dextrose as anticoagulant (1:6 volumes of blood) and washed platelets were prepared in the presence of 0.2 $\mu$M PGI$_2$, 50 U/ml heparin and 0.01 mg/ml apyrase and incubated with $[^{3}H]AA$ (1 $\mu$Ci/ml of platelet suspension) at 37°C for 1 h. The unincorporated precursor or was removed by further washings. Finally, WLP were resuspended at 250,000/µl in a convenient volume of Tyrode-BSA buffer (pH 7.4) without Ca$^{2+}$ and Mg$^{2+}$.

2.3. Stimulation of washed labelled platelets

0.5 ml WLP suspension was stimulated and with thrombin 0.5 U/ml for 3 min or with 21 mM AlF$_4^-$ (NaF and AlCl$_3$ solution in a 5000:1 molar ratio) for 20 min under continuous stirring at 1,100 rpm [7]. When indicated, WLP were preincubated with PMA (100 nM for 5 min) or staurosporine (200 nM for 4 min) or H-7 (200 $\mu$M for 4 min) or calphostin C (2 $\mu$M for 4 min) or okadaic acid (1 $\mu$M for 4 min or for 10 min) before stimulation with AlF$_4^-$ or with thrombin. Platelet aggregation was monitored by photometric method (Aggre- corder, Menarini, Milan, Italy). At the end of the stimulation period, 0.1 ml platelet suspension were taken for $\beta$-TG determination and the remaining amount for lipid analysis.

2.4. Extraction and analysis of lipids

Lipids were extracted and AA and phosphatidic acid (PA) were isolated by TLC as previously described [7]. The corresponding areas were scraped off and their radioactivity determined by liquid scintillation counting (Emulsifier-safe, Packard Instrument Co., IL) and quenching corrected by external standardization.

2.5. Determination of $\beta$-thromboglobulin

0.1 ml platelet suspensions were immediately centrifuged at 12,000 x g for 2 min and the supernatant stored at -80°C for subsequent assay of $\beta$-TG which was performed by ELISA (Boehringer, Mannheim, Germany) [16].

2.6. Statistical analysis

Unless differently indicated, statistical analysis of the data was carried out by ANOVA followed by multiple comparison (Sheffe's test).

3. Results

Human platelets, prelabelled with $[^{3}H]AA$ and stimulated with thrombin or fluoroaluminate, released this fatty acid from membrane phospholipids, liberated $\beta$-TG from granules and aggregated (Table 1). Concentrations of agonists and times of stimulation were chosen to give comparable biochemical and functional effects [7]. The exposure of platelets to 100 nM PMA for 5 min did not increase the radioactivity of AA although induced $\beta$-TG release (near 40% of total content) and platelet aggregation. Since PMA activates PKC, this result indicates that, in intact platelets, this enzyme does not induce direct activation of PLA$_2$ but it participates to secretion and aggregation. The preincubation of platelets with PMA (100 nM) did not modify significantly the release of $[^{3}H]AA$ from thrombin- or AlF$_4^-$-stimulated platelets (Table 1).

The preincubation of WLP with staurosporine (200 nM) or with H-7 (200 $\mu$M) increased the release of labelled AA by 70-80%, reduced the release of $\beta$-TG by more than 50% and blocked completely the aggregation when platelets were stimulated with AlF$_4^-$ (Table 2). A rather different pattern of effects was observed instead when platelets were stimulated with 0.5 U/ml thrombin. In fact, the treatment with staurosporine or H-7 had virtually no effect on the radioactivity of free AA or on the release of $\beta$-TG or platelet aggregation (Table 2).

When platelets were preincubated with calphostin C (2 $\mu$M) for 4 min before stimulation with AlF$_4^-$, aggregation was completely blocked, $\beta$-TG release was inhibited by nearly 60% (total content of $\beta$-TG = 9222 ± 643 (n = 6) ng/ml; AlF$_4^-$ (21 mM) = 3327 ± 1696 (n = 17); calphostin C (2 $\mu$M) + AlF$_4^-$ (21 mM) = 1427 ± 316 (n = 4)) but the liberation of $[^{3}H]AA$ was increased by nearly 100% (Fig. 1). Under identical conditions, the release of $[^{3}H]AA$ tended to increase in thrombin-stimulated WLP preincubated with calphostin (Fig. 1) whereas the release of $\beta$-TG and platelet aggregation were slightly reduced (data not shown). Calphostin C induced by itself an increase of the radioactivity associated with free AA (Fig. 1) but had no effect on platelet aggregation (data not shown).

Table 1

<table>
<thead>
<tr>
<th>Treatment</th>
<th>AA production (nCi/10$^8$ platelets)</th>
<th>$\beta$-TG release (ng/ml)</th>
<th>% of maximal aggregation</th>
</tr>
</thead>
<tbody>
<tr>
<td>Resting</td>
<td>10.1 ± 1.5 (22)</td>
<td>381 ± 79 (7)</td>
<td>0</td>
</tr>
<tr>
<td>PMA (100 nM)</td>
<td>12.1 ± 1.6 (10)</td>
<td>3588 ± 646 (9)</td>
<td>55 ± 5 (9)</td>
</tr>
<tr>
<td>AlF$_4^-$</td>
<td>50.7 ± 3.4 (22)$^b$</td>
<td>3327 ± 411 (17)$^b$</td>
<td>51 ± 3 (8)</td>
</tr>
<tr>
<td>PMA 100 nM + AlF$_4^-$</td>
<td>42.1 ± 4.2 (16)</td>
<td>3678 ± 528 (17)$^b$</td>
<td>52 ± 2 (9)</td>
</tr>
<tr>
<td>Thrombin</td>
<td>65.9 ± 5.6 (18)$^b$</td>
<td>4028 ± 396 (16)$^b$</td>
<td>55 ± 2 (10)</td>
</tr>
<tr>
<td>PMA 100 nM + thrombin</td>
<td>56.9 ± 8.2 (10)$^b$</td>
<td>4062 ± 590 (8)$^b$</td>
<td>55 ± 3 (8)</td>
</tr>
</tbody>
</table>

Washed labelled platelets (WLP) were prepared and $[^{3}H]AA$, $\beta$-TG release and aggregation were determined as described in section 2. WLP were stimulated with AlF$_4^-$ (21.1 mM NaF + 4.2 $\mu$M AlCl$_3$) for 20 min or with 0.5 U/ml thrombin for 3 min. When indicated WLP were preincubated with 100 nM PMA for 5 min before stimulation. Data are the means ± S.E.M. Total content (Triton X-100 extraction) of $\beta$-TG in WLP was 9222 ± 287 (n = 5) ng/ml.

$^b$P < 0.05 vs. resting. Number of determinations between brackets.
Fig. 1. The effect of calphostin C on the production of arachidonic acid in intact human platelet stimulated with fluoroulate or thrombin. Washed labelled platelet (WLP) preparations and [3H]AA release determination were performed as described in section 2. WLP were stimulated with AIF4 (21.1 mM NaF+4.2 ~M AICl3) for 20 min (F) or with 0.5 U/ml thrombin for 3 min (T). When indicated WLP were preincubated before stimulation with calphostin C (2 M) for 4 min (C). Data are the means ± S.E.M. from at least two experiments in duplicate. R = resting platelets. *P < 0.01 vs. R; §P < 0.01 vs. C; #P < 0.01 vs. F.

As shown in Table 3, the stimulation of WLP with AIF4 or with thrombin caused nearly 100% increase of phosphatidic acid (PA) radioactivity which was not significantly affected by the exposure to staurosporine or to H-7. On the other hand, calphostin C reduced the increase of the radioactivity of PA induced by AIF4 but had no effect on that induced by thrombin (Table 3).

Pretreatment of platelets with 1 μM okadaic acid for 4 min before stimulation with AIF4 induced a marked decrease of AA liberation, β-TG release (Fig. 2) and completely inhibited platelet aggregation. The same treatment did not cause significant changes of these parameters in thrombin-stimulated platelets, whereas they were reduced by 20–30% when preincubation with okadaic acid was prolonged to 10 min (data not shown).

4. Discussion

The release of AA from stimulated platelets is attributed essentially to the activation of PLA2. It remains to be established whether a single enzyme, likely cPLA2, is the target of multiple signals converging to a common mechanism of enzyme activation or if different platelet stimuli activate different PLA2s through distinct pathways.

In the present study, we have stimulated human platelets by a direct and unspecific G-protein activator (AIF4) which induces platelets to aggregate, to release β-TG from α-granules and to produce free AA by the hydrolysis of membrane phospholipids, through a mechanism which differs from that initiated by thrombin, at least in part [7].

We have assessed the involvement of PKC in these phenomena by using an activator (PMA) or several inhibitors (staurosporine, H-7 or calphostin C) of the enzyme. Other Authors have shown that, in permeabilized rabbit platelets, PMA potentiates AA release induced by GTP-S or AIF4 in the presence of 100 μM external Ca2+ concentration while staurosporine had an inhibitory effect [12]. On the contrary, our results have shown that the exposure of intact platelets, resuspended in a medium without added Ca2+, to PMA did not increase the release of AA induced by AIF4 or with thrombin. The discrepancy between our and previous studies cannot be attributed to a failure of PMA to activate PKC since β-TG release from α-granules, a PKC-mediated process, took place (Table 1), and it was reduced by staurosporine (data not shown). Thus, it can be suggested that the potentiation by PKC of the G-protein mediated activation of PLA2, reported with rabbit permeabilized platelets [12], requires a relative high Ca2+ concentration which might be provided by an influx from the external medium. This hypothesis is supported by the observation that, in human platelets stimulated by Ca2+-ionophore, the release of AA is also potentiated by PMA and inhibited by staurosporine and H-7 [13].

### Table 2

The effect of protein kinase inhibitors on the production of arachidonic acid, on the release of β-TG and on aggregation in intact human platelet stimulated with fluoroulate or thrombin

<table>
<thead>
<tr>
<th>Treatment</th>
<th>AA production (nCi/10^9 platelets)</th>
<th>β-TG release (ng/ml)</th>
<th>Aggregation (% of control)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Resting</td>
<td>10.1 ± 1.5 (22)</td>
<td>381 ± 79 (17)</td>
<td>0</td>
</tr>
<tr>
<td>Staurosporine</td>
<td>8.2 ± 0.7 (5)</td>
<td>475 ± 14 (2)</td>
<td>0 (5)</td>
</tr>
<tr>
<td>H-7</td>
<td>6.6 ± 1.4 (2)</td>
<td>-</td>
<td>0 (2)</td>
</tr>
<tr>
<td>AIF4</td>
<td>50.7 ± 3.4 (22)</td>
<td>3327 ± 411 (17)</td>
<td>100</td>
</tr>
<tr>
<td>Staurosporine + AIF4</td>
<td>88.5 ± 9.6 (14)</td>
<td>1528 ± 206 (12)</td>
<td>6.2 ± 2.1 (12)</td>
</tr>
<tr>
<td>H-7 + AIF4</td>
<td>93.8 ± 14.7 (6)</td>
<td>1207 ± 208 (6)</td>
<td>19.2 ± 11.8 (6)</td>
</tr>
<tr>
<td>Thrombin</td>
<td>113.8 ± 6.4 (5)</td>
<td>3042 ± 222 (8)</td>
<td>100</td>
</tr>
<tr>
<td>Staurosporine + Thrombin</td>
<td>98.9 ± 8.3 (6)</td>
<td>3928 ± 282 (8)</td>
<td>100.9 ± 3.3 (10)</td>
</tr>
<tr>
<td>H-7 + Thrombin</td>
<td>91.0 ± 4.7 (6)</td>
<td>3019 ± 584 (6)</td>
<td>98 ± 4 (4)</td>
</tr>
</tbody>
</table>

WLP were stimulated with AIF4 (21.1 mM NaF + 4.2 μM AICl3) for 20 min or with 0.5 U/ml thrombin for 3 min. When indicated WLP were alternatively preincubated before stimulation with staurosporine 200 nM for 4 min or H-7 200 μM for 4 min. Data are the mean ± S.E.M. with number of determinations between brackets. Total content (Trition X-100 extraction) of β-TG in WLP was 9222 ± 287 (n = 5) ng/ml.

*P < 0.05 vs. AIF4.

*P < 0.05 vs. resting.
Most interestingly, we have demonstrated that staurosporine or H-7, unspecific PKC inhibitors, and the more selective calphostin C, increased the amount of AA released by AIF₄ while abolished the release of β-TG (Table 2). These results indicate that, in intact human platelets, there is a G-protein mediated mechanism for the activation of a PLA₂ which is downregulated by PKC or another protein kinase(s) sensible mediated by protein kinase(s), while abolished the release of β-TG (Table 2). These results indicate that, in intact human platelets, there is a G-protein mediated mechanism for the activation of a PLA₂ which is downregulated by PKC or another protein kinase(s) sensible to phosphorylation, H-7 or calphostin C. Thus, this enzyme must be different from that described in previous studies [12,13].

AIF₄-stimulated PLA₂ is likely to be Ca²⁺-independent [17] or to require low Ca²⁺ concentration. In fact, the stimulation of intact human platelets, resuspended in a Ca²⁺-free medium, increases intracellular [Ca²⁺] up to 500 nM [18], a concentration which might be sufficient to activate cPLA₂. If cPLA₂ is the enzyme which is activated in our experimental conditions, we can exclude that staurosporine, H-7 and calphostin C act on MAPK or other proline-directed kinases, which have PLA₂ as substrate, because the latter enzyme is more active when phosphorylated [10,11].

Differently from what observed with AIF₄, PKC inhibitors have no effect on the formation of labelled AA, β-TG release and aggregation induced by 0.5 U/ml thrombin (Table 2). This discrepancy might be due to differences in the signal transduction pathways leading to PLA₂ activation in receptor-stimulated versus AIF₄ (G-protein)-activated platelets. Alternatively, it is possible that the two stimuli lead to the activation of different enzymes.

Calphostin C, differently from staurosporine and H-7, induced the direct release of [³H]AA. A similar finding has been reported in human neutrophils where this compound induced a dose-dependent release of AA [19]. In addition, calphostin C inhibits the labelling of PA in AIF₄-stimulated platelets (Table 3). These effects are not probably related to the inhibition of PKC because staurosporine or H-7 were not able to induce a direct AA release or to reduce PA formation. These observations further encourage caution in considering calphostin C simply a PKC-inhibitor.

We have found that the treatment of intact platelets with okadaic acid, a specific inhibitor of protein phosphatases 1 and 2A, causes a marked reduction of AA liberation, β-TG release and prevents the aggregation in AIF₄-stimulated platelets and, to a lesser extent, in thrombin-stimulated platelets. Although several studies have been reported on the effect of inhibitors of protein phosphatases on platelet release and aggregation [20,21], so far no information is available concerning AA release in intact cells. Okadaic acid inhibits thrombin induced platelet activation through a potentiation of the inhibition of phospholipase C produced by PKA and PKC [22]. Since the release of AA in AIF₄-stimulated platelets does not depend on PLC activation [7], in our experiments the effects of okadaic acid must be produced by another mechanism.

In conclusion, we suggest the existence in intact human platelets of a G-protein mediated mechanism for the activation of a PLA₂ which is regulated by phosphorylation-dephosphorylation reactions where a protein kinase(s), sensible to PKC inhibitors, should exert a negative modulation of PLA₂ activity while a protein phosphatase(s), sensible to okadaic acid, should have the opposite effect. The nature of these enzymes and their location in the cascade of events leading to PLA₂ activation remains to be established.

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References