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Protein kinase C inhibitors enhance G-protein induced phospholipase A_2 activation in intact human platelets

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Abstract Washed intact human platelets were prelabelled with [^{3}H]arachidonic acid ([^{3}H]AA) and stimulated with thrombin or with AlF_4^-, a known unspecific activator of G-proteins. Both stimuli induced the liberation of [^{3}H]AA, the release of β-thromboglobulin (β-TG) and platelet aggregation. PMA did not induce liberation of [^{3}H]AA although it induced β-TG release and aggregation; preincubation with PMA did not modify significantly the amounts of [^{3}H]AA and β-TG released by thrombin or AlF_4^-. Different inhibitors of PKC (staurosporine, H-7 and calphostin C) increased the release of [^{3}H]AA and inhibited β-TG release and aggregation induced by AlF_4^- but they had no effect when platelets were stimulated with thrombin (0.5 U/ml). Calphostin C was able to release [^{3}H]AA by itself without inducing aggregation or β-TG release. Okadaic acid (a serine/threonine phosphoprotein phosphatase inhibitor) greatly inhibited the release of [^{3}H]AA, β-TG and aggregation in AlF_4^--stimulated platelets. These results indicate the presence of a G-protein mediated mechanism for the activation of a platelet phospholipase A_2 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 A_2 activation (human platelets); Protein kinase C inhibitors; G-Protein; β-Thromboglobulin release; Protein phosphatase

1. Introduction

Type II phospholipase A_2 ($sPLA_2$) and type IV phospholipase A_2 ($cPLA_2$) have been detected and characterized in human platelets. The former enzyme is released from stimulated platelets and it is identical to the PLA_2 present in rheumathoid 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 $sPLA_2$ contributes to the liberation of arachidonic acid (AA) in stimulated platelets is still matter of debate. On the other hand, the involvement of $cPLA_2$ 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 Ca^{2+} and phosphorylation for full activation [5]. Since the activation of $cPLA_2$ 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 (AlF_4^-), with those of thrombin on PLA_2 activation as well as aggregation and release of granule contents in intact human platelets. We have demonstrated that PLA_2 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 $cPLA_2$ 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 $cPLA_2$ 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 $cPLA_2$ cannot be ruled out [10].

In other cell types, PKC-dependent and PKC-independent mechanisms for the activation of MAPK and consequently of $cPLA_2$ have been proposed [11].

In platelets, the role of PKC in G-protein mediated activation of PLA_2 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 Ca^{2+} addition [12]. In intact human platelets, PMA potentiates Ca^{2+}-ionophore induced AA release [13] whereas it inhibits the formation of thromboxane B_2 (TxB_2) caused by AlF_4^- addition [14].

AlF_4^- induces platelet activation by mechanisms not fully elucidated. This compound (10 mM or less) produces diacylglycerol and TxB_2, without activating PLC, and increases PKC activity without an apparent increase of intracellular Ca^{2+} [15]. At higher concentrations (20 mM or more), AlF_4^- activates PLC and liberates TxB_2 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 PLA_2, 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

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Abbreviations: AA, arachidonic acid; H-7, 1-(5-isoquinolinylsulfonyl)-2-methylpiperazine; MAPK, mitogen-activated protein kinase; PA, phosphatidic acid; PGI_2, prostacyclin; PKC, protein kinase C; PLC, phospholipase C; PMA, phorbol 12-myristate 13-acetate; $cPLA_2$, cytosolic phospholipase A_2; $sPLA_2$, secretable phospholipase A_2; β-TG, β-thromboglobulin; TxB_2 , thromboxane B_2; WLP, washed labelled platelets.

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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-^{3}H]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 μM PGI_2, 50 U/ml heparin and 0.01 mg/ml apyrase and incubated with [^{3}H]AA (1 μCi/ml of platelet suspension) at 37°C for 1 h. The unincorporated precursor 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 μM for 4 min) or calphostin C (2 μM for 4 min) or okadaic acid (1 μM for 4 min or for 10 min) before stimulation with AlF_4^- or with thrombin. Platelet aggregation was monitored by photometric method (Aggregorder, Menarini, Milan, Italy). At the end of the stimulation period, 0.1 ml platelet suspension were taken for β-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 β-thromboglobulin

0.1 ml platelet suspensions were immediately centrifuged at 12,000×g for 2 min and the supernatant stored at −80°C for subsequent assay of β-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 β-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 β-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 μM) increased the release of labelled AA by 70–80%, reduced the release of β-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 were 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 β-TG or platelet aggregation (Table 2).

When platelets were preincubated with calphostin C (2 μM) for 4 min before stimulation with AlF_4^-, aggregation was completely blocked, β-TG release was inhibited by nearly 60% (total content of β-TG = 9222 ± 643 (n = 6) ng/ml; AlF_4^- (21 mM) = 3327 ± 1696 (n = 17); calphostin C (2 μM) + 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 β-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
The effect of fluoroaluminate, thrombin or PMA on the production of arachidonic acid, on the release of β-TG and aggregation in intact human platelets

Treatment	AA production (nCi/10^9 platelets)	β-TG release (ng/ml)	% of maximal aggregation
Resting	10.1 ± 1.5 (22)	381 ± 79 (7)	0
PMA (100 nM)	12.1 ± 1.6 (10)	3588 ± 646 (9)	55 ± 5 (9)
AlF_4^-	50.7 ± 3.4 (22)[b]	3327 ± 411 (17)[b]	51 ± 3 (8)
PMA 100 nM + AlF_4^-	42.1 ± 4.2 (16)	3678 ± 528 (17)[b]	52 ± 2 (9)
Thrombin	65.9 ± 5.6 (18)[b]	4028 ± 396 (16)[b]	55 ± 2 (10)
PMA 100 nM + thrombin	56.9 ± 8.2 (10)[b]	4062 ± 590 (8)[b]	55 ± 3 (8)

Washed labelled platelets (WLP) were prepared and [^{3}H]AA, β-TG release and aggregation were determined as described in section 2. WLP were stimulated with AlF_4^- (21.1 mM NaF + 4.2 μM $AlCl_3$) 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 β-TG in WLP was 9222 ± 287 (n = 5) ng/ml.
[b] $P < 0.05$ vs. resting. Number of determinations between brackets.

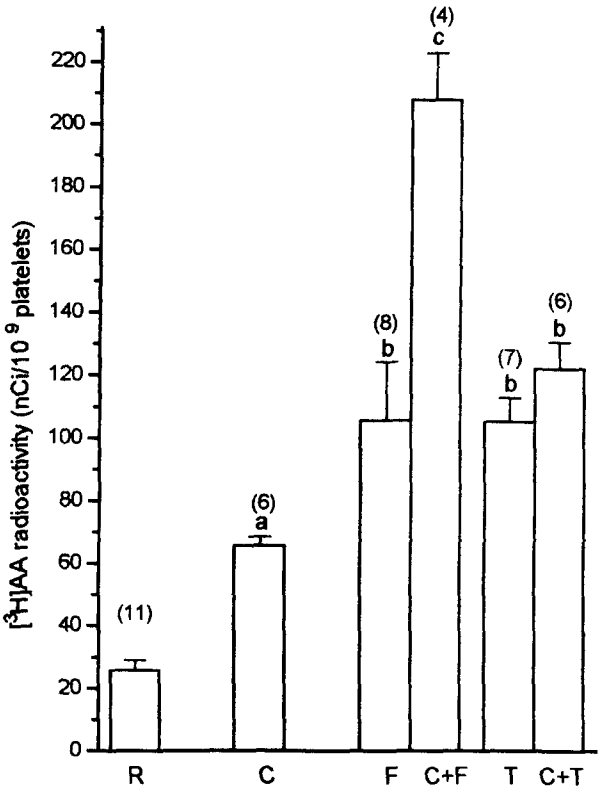

Fig. 1. The effect of calphostin C on the production of arachidonic acid in intact human platelet stimulated with fluoraluminate or thrombin. Washed labelled platelet (WLP) preparations and [^{3}H]AA release determination were performed as described in section 2. WLP were stimulated with AlF_4^- (21.1 mM NaF + 4.2 μM $AlCl_3$) 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; $^{\S}P < 0.01$ vs. C; $^{\#}P < 0.01$ vs. F.

As shown in Table 3, the stimulation of WLP with AlF_4^- 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 AlF_4^- 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 AlF_4^- 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 PLA_2. It remains to be established whether a single enzyme, likely $cPLA_2$, is the target of multiple signals converging to a common mechanism of enzyme activation or if different platelet stimuli activate different PLA_2s through distinct pathways.

In the present study, we have stimulated human platelets by a direct and unspecific G-protein activator (AlF_4^-) 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 AlF_4^- in the presence of 100 μM external Ca^{2+} concentration while staurosporine had an inhibitory effect [12]. On the contrary, our results have shown that the exposure of intact platelets, resuspendended in a medium without added Ca^{2+}, to PMA did not increase the release of AA induced by AlF_4^- 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 PLA_2, reported with rabbit permeabilized platelets [12], requires a relative high Ca^{2+} 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 Ca^{2+}-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 fluoroaluminate or thrombin

Treatment	AA production (nCi/10^9 platelets)	β-TG release (ng/ml)	Aggregation (% of control)
Resting	10.1 ± 1.5 (22)	381 ± 79 (17)	0
Staurosporine	8.2 ± 0.7 (5)	475 ± 14 (2)	0 (5)
H-7	6.6 ± 1.4 (2)	–	0 (2)
AlF_4^-	50.7 ± 3.4 (22)^b	3327 ± 411 (17)^b	100
Staurosporine + AlF_4^-	88.5 ± 9.6 (14)^a	1528 ± 206 (12)^a	6.2 ± 2.1 (12)
H-7 + AlF_4^-	93.8 ± 14.7 (6)^a	1207 ± 208 (6)^a	19.2 ± 11.8 (6)
Thrombin	113.8 ± 6.4(5)^b	3042 ± 222 (8)^b	100
Staurosporine + Thrombin	98.9 ± 8.3 (6)	3928 ± 282 (8)	100.9 ± 3.3 (10)
H-7 + Thrombin	91.0 ± 4.7 (6)	3019 ± 584 (6)	98 ± 4 (4)

WLP were stimulated with AlF_4^- (21.1 mM NaF + 4.2 μM $AlCl_3$) 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 (Triton X-100 extraction) of β-TG in WLP was 9222 ± 287 ($n = 5$) ng/ml.
$^a P < 0.05$ vs. AlF_4^-.
$^b P < 0.05$ vs. resting.

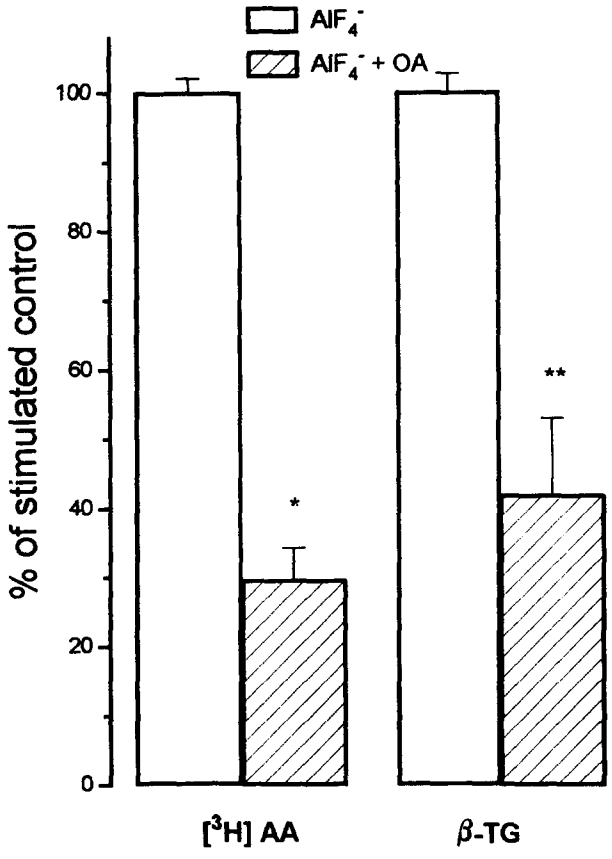

Fig. 2. Effect of okadaic acid on AA liberation, β-TG release and aggregation in intact human platelets. WLP were preincubated with 1 μM okadaic acid for 4 min (OA) and stimulated with AlF_4^- (21.1 mM NaF + 4.2 μM $AlCl_3$) for 20 min. Data are the mean ± S.E.M. from 3 experiments in duplicate. $^*P < 0.0001$ and $^{**}P < 0.05$ vs. stimulated control (Student's *t*-test).

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 AlF_4^- 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 PLA_2 which is downregulated by PKC or another protein kinase(s) sensible to staurosporine, H-7 or calphostin C. Thus, this enzyme must be different from that described in previous studies [12,13].

AlF_4^--stimulated PLA_2 is likely to be Ca^{2+}-independent [17] or to require low Ca^{2+} concentration. In fact, the stimulation of intact human platelets, resuspended in a Ca^{2+}-free medium, increases intracellular $[Ca^{2+}]$ up to 500 nM [18], a concentration which might be sufficient to activate $cPLA_2$. If $cPLA_2$ 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 $cPLA_2$ as substrate, because the latter enzyme is more active when phosphorylated [10,11].

Differently from what observed with AlF_4^-, 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 PLA_2 activation in receptor-stimulated versus AlF_4^- (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 [^{3}H]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 AlF_4^--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 AlF_4^--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 AlF_4^- -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 PLA_2 which is regulated by phosphorylation–dephosphorylation reactions where a protein kinase(s), sensible to PKC inhibitors, should exert a negative modulation of PLA_2 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 PLA_2 activation remains to be established.

Table 3
The effect of fluoroaluminate, thrombin and of three different protein kinase inhibitors on the formation of radioactive phosphatidic acid (PA)

Treatment	PA radioactivity (nCi/10^9 platelets)
Resting	6.18 ± 3.17 (20)
AlF_4^-	18.6 ± 7.87 (21)[a]
Staurosporine (200 nM) + AlF_4^-	14.8 ± 5.7 (12)
H-7 (200 μM) + AlF_4^-	12.7 ± 1.5 (4)
Calphostin C (2 μM) + AlF_4^-	8.7 ± 2.0 (5)[b]
Thrombin	15.7 ± 3.2 (15)[a]
Staurosporine (200 nM) + Thrombin	14.1 ± 3.2 (8)
H-7 (200 μM) + Thrombin	11.7 ± 3.4 (6)
Calphostin C (2 μM) + Thrombin	11.6 ± 6.8 (4)

WLP were stimulated with AlF_4^- (21.1 mM NaF + 4.2 μM $AlCl_3$) for 20 min or with 0.5 U/ml thrombin for 3 min. Data are the mean ± S.E.M.
[a] $P < 0.05$ vs. resting.
[b] $P < 0.05$ vs. AlF_4^-.

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