Thrombin and C-kinase activators potentiate calcium-stimulated arachidonic acid release in human platelets

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Human platelets were depleted of intracellular Ca^{2+} and then made selectively permeable to external Ca^{2+} by addition of the ionophore ionomycin. In this cell system a rapid release of arachidonic acid was seen in direct response to added Ca^{2+} at concentrations corresponding to cytosolic Ca^{2+} levels measured in thrombin-stimulated platelets. Thrombin and other activators of Ca^{2+} /phospholipid-dependent protein kinase (C-kinase) potentiated the Ca^{2+} -stimulated arachidonic acid release while exerting little or no effect in the absence of added Ca^{2+} . Agents which increase (R59022) or decrease (isoquinolinesulphonylmethylpiperazine) the activation of C-kinase correspondingly enhanced or inhibited, respectively, the potentiation of arachidonic acid release caused by thrombin. These results support the hypothesis that arachidonic acid release in human platelets is regulated by a co-operative action between intracellular Ca^{2+} and C-kinase.

INTRODUCTION

In common with many other cell types, human platelets respond to Ca^{2+} -mobilizing stimulatory agonists by rapidly hydrolysing membrane phospholipids, thereby generating a variety of important intracellular and extracellular messengers. The action of phospholipase C on phosphoinositides leads to a transient accumulation of the intracellular signals inositol trisphosphate and diacylglycerol [1–4]. These products in turn are thought to evoke an increase in cytosolic Ca^{2+} level and the activation of C-kinase, respectively, thus mobilizing dual branches of the Ca^{2+} messenger system [5,6]. Both branches of this pathway may interact synergistically to initiate platelet responses, including secretion and aggregation [7]. Activated platelets also produce free arachidonic acid (AA), the precursor for thromboxane A_2 and other eicosanoids, which escape the cell to function as intercellular messengers. Although the regulatory mechanisms which underlie the AA-release process are not clearly understood, a central role for Ca^{2+} is indicated by the ability of Ca^{2+} ionophores (A23187, ionomycin) to evoke the liberation of this fatty acid [8,9]. We have recently shown that activators of C-kinase enhance the release of AA in platelets stimulated by A23187, suggesting that C-kinase could function co-operatively with Ca^{2+} to regulate platelet AA mobilization [10].

In this communication we show that a novel system of intact, Ca^{2+} -depleted, Ca^{2+} -permeable human platelets can be stimulated by physiological (0.3–6.0 μM) levels of Ca^{2+} to mobilize AA. These results provide direct evidence that AA release may occur in response to the rapid rise in the cytoplasmic $[\text{Ca}^{2+}]$ seen in platelets activated by physiological agonists [11,12]. Further, the Ca^{2+} -stimulated release of AA was greatly potentiated by the platelet agonist thrombin as well as other activators of C-kinase, suggesting a synergistic interaction between Ca^{2+} and C-kinase for regulation of human platelet AA mobilization.

MATERIALS AND METHODS

Materials

[^3H]AA, 210 Ci/mmol, was purchased from Amersham. Human thrombin (3200 units/mg) was obtained from United States Biochemical Corp., Cleveland, OH, U.S.A. 4 β -Phorbol 12-myristate-13-acetate (PMA), 4 β -phorbol 12,13-dibutyrate and 4 α -PMA were from LC Services Corp., Woburn, MA, U.S.A. (concentrated stock solutions were prepared in dimethyl sulphoxide). 1-Oleoyl-2-acetyl-*sn*-glycerol (OAG) was from Avanti Polar Lipids, Birmingham, AL, U.S.A. (for addition to platelets the lipid was suspended in incubation medium as previously described [10]). R59022 was purchased from Janssen Pharmaceutica, Piscataway, NJ, U.S.A. and dissolved in ethanol. Isoquinolinesulphonylmethylpiperazine (H-7) was obtained from Seikagaku America Inc., St. Petersburg, FL, U.S.A. Ca^{2+} ionophores A23187 (from United States Biochemical) and ionomycin (from Calbiochem, San Diego, CA, U.S.A.) were dissolved in dimethyl sulphoxide. The concentrations of dimethyl sulphoxide and ethanol in cell incubations never exceeded 0.2% and 0.1% (v/v), respectively, and had no apparent effect of their own on AA release in control incubations. All other reagents were purchased from Sigma or Fisher (St. Louis, MO, U.S.A.).

Methods

For most experiments, fresh human platelet concentrates were purchased from the American Red Cross Mid-Missouri Blood Center, Columbia, MO, U.S.A. Essentially identical results were obtained with platelets prepared in the laboratory from blood drawn from normal human volunteers. Platelets were harvested and washed by centrifugation as previously described [10]. Washed platelets were resuspended at a cell density of $2 \times 10^9/\text{ml}$ in a medium (solution A) containing 145 mM-NaCl, 5 mM-KCl, 5 mM-EGTA, 5.5 mM-dextrose, 0.2 mg

Abbreviations used: AA, arachidonic acid; H-7, isoquinolinesulphonylmethylpiperazine; OAG, 1-oleoyl-2-acetyl-*sn*-3-glycerol; PMA, 4 β -phorbol 12-myristate-13-acetate.

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Fig. 1. Effects of thrombin and PMA on Ca^{2+} -stimulated $[^3\text{H}]\text{AA}$ release

Ca^{2+} -depleted platelets were stimulated with the indicated free Ca^{2+} concentrations in the absence (control) or presence of thrombin (2 units/ml) or PMA (100 nM). Incubations were terminated after 10 min, and $[^3\text{H}]\text{AA}$ release into the medium was measured as described in the Materials and methods section. Results are means $\pm$ S.D. (indicated by bars unless smaller than the symbols) of triplicate samples from a typical experiment. Basal radioactivity in the medium (in the absence of added Ca^{2+}) was 5280 ± 79 c.p.m. This value, which did not change during the time course of the experiment, was subtracted from all values shown in the Figure. ○, Control; ●, thrombin; ▲, PMA.

of fatty-acid-free bovine serum albumin/ml, and 10 mM-Hepes, pH 7.4. To deplete cellular Ca^{2+} , 2 μM -ionomycin was added and the platelet suspension was incubated for 20 min at 37 °C with gentle shaking. In some experiments, platelets were treated with 2 μM -A23187 instead of ionomycin; results were similar in both cases. Platelets were then harvested and washed to remove released Ca^{2+} . This procedure reduced total platelet calcium by 80–90 % and magnesium by 20–30 % (as determined by atomic absorption spectroscopy [13]). Leakage of lactate dehydrogenase (assayed according to Brass [14]) during ionomycin treatment did not exceed that of control samples (1.0–1.5 % of total cell activity). Ca^{2+} -depleted platelets retained the ability to aggregate in response to thrombin (0.2 unit/ml) when 1 mM- CaCl_2 was added back to the medium (not shown), indicating that Ca^{2+} depletion does not abolish platelet responsiveness provided external Ca^{2+} is present.

In order to label platelet phospholipids with $[^3\text{H}]\text{AA}$, Ca^{2+} -depleted platelets were resuspended in a medium identical to solution A, except that it contained 1 mM-EGTA, 1 mM- MgCl_2 , 10 mg of fatty-acid-free bovine serum albumin/ml and 2 μCi of $[^3\text{H}]\text{AA}$ /ml. The platelet suspension was incubated for 60 min at 37 °C, resulting in incorporation of 70–90 % of added radiolabel (essentially identical to non-depleted platelets, cf. [10]). Labelled platelets were washed and resuspended at 5×10^8 cells/ml in final incubation medium composed of

135 mM- NaCl , 5 mM- KCl , 1 mM- MgCl_2 , 0.5 mM-EGTA, 0.5 mM-EDTA, 5.5 mM-dextrose, and 25 mM-Hepes, pH 7.40, at 37 °C. All of the experiments shown were carried out with an incubation medium containing 1 mM total $[\text{Mg}^{2+}]$. However, closely similar results were obtained when the total $[\text{Mg}^{2+}]$ was varied from 0.4 to 4.0 mM (corresponding to a calculated free $[\text{Mg}^{2+}]$ of 0.01–3.00 mM). Platelets were rendered selectively permeable to Ca^{2+} by re-addition of 2 mM-ionomycin and pre-incubated for 5 min with or without various drugs as indicated in the Figure legends. CaCl_2 (buffered by EGTA/EDTA in the medium) was added to yield the desired free $[\text{Ca}^{2+}]$, calculated as described by Fabiato & Fabiato [15]. CaCl_2 was prepared by dissolution of CaCO_3 in dilute HCl because of the uncertain degree of hydration of commercially available CaCl_2 . Medium pH was not detectably altered by addition of CaCl_2 solutions. Thrombin, when present, was added simultaneously with CaCl_2 .

For measurements of $[^3\text{H}]\text{AA}$ release, incubations (0.5 ml samples of platelet suspension) were terminated by adding 0.1 ml of 1 M-formaldehyde/50 mM-EDTA (pH 7); the samples were then centrifuged for 1 min at 13000 g. A portion of the cell-free supernatant, containing released AA plus cyclo-oxygenase- and lipoxygenase-derived metabolites, was assayed for ^3H content by scintillation counting. $[^3\text{H}]\text{Phospholipids}$ were extracted according to Bligh & Dyer [16] and separated by t.l.c. as previously described [17]. Phospholipid bands were identified by co-migration with authentic standards following visualization with toluidinylnaphthalene sulphinate spray reagent [18], scraped from the plates, and then added to a scintillation cocktail composed of Scintiverse (Fisher Chemical Corp.)/methanol/water (20:1.5:1, by vol.). Radioactivity was determined with a Beckman LS 1801 liquid-scintillation counter. Each experiment was carried out in triplicate and was replicated with platelet preparations from three or more donors. The statistical significance of differences between control and drug treatments was determined by Student's *t* test for paired comparisons.

RESULTS AND DISCUSSION

In order to study directly the role of Ca^{2+} in AA release while avoiding the leakage of low-molecular-mass intracellular components which occurs in detergent- or electrically permeabilized cells, human platelets were depleted of their intracellular Ca^{2+} store and then made selectively permeable to external Ca^{2+} by addition of ionomycin. Ca^{2+} was added to the medium in the form of Ca^{2+} /EGTA/EDTA buffers calculated [15] to yield a free $[\text{Ca}^{2+}]$ over the range of 0.1–10.0 μM , corresponding to cytosolic Ca^{2+} levels previously measured in control and thrombin-stimulated platelets by quin2 or aequorin techniques [11,12,19]. As shown in Fig. 1, Ca^{2+} at concentrations within the physiological range evoked a release of $[^3\text{H}]\text{AA}$ from platelets into the medium. This response to Ca^{2+} was rapid, displaying a time course similar to that for thrombin-stimulated AA release in control cells (not shown). The threshold $[\text{Ca}^{2+}]$ was approx. 0.3 μM and a maximal AA release was seen at a $[\text{Ca}^{2+}]$ of between 3 and 6 μM in different experiments. Thus, Ca^{2+} at levels attained intracellularly in response to thrombin is a sufficient stimulus for AA release in platelets. It is notable that AA release in this study

Table 1. Effect of phorbol esters, mezerein and OAG, on Ca^{2+} -stimulated [^3H]AA release

Ca^{2+} -depleted platelets were pre-incubated for 5 min with the indicated drugs (at 100 nM except for OAG, which was used at 50 μM) and then stimulated with 3 μM free Ca^{2+} for an additional 10 min. Results are means $\pm$ S.D. of triplicate determinations from a typical experiment. Basal radioactivity (in the absence of added Ca^{2+}) was 1920 ± 710 c.p.m.; this did not change over the time course of the experiment. None of the pretreatments affected AA release in the absence of added Ca^{2+} .

Pretreatment drug	AA release (c.p.m.)
None (control)	9370 ± 870
PMA	29000 ± 1160
4 α -PMA	9020 ± 207
4 β -Phorbol 12,13-dibutyrate	28500 ± 884
Mezerein	27600 ± 690
OAG	21100 ± 1070

displays a very similar [Ca^{2+}] dependency to that reported for serotonin secretion in electrically-permeabilized platelets [20,21].

Both the platelet agonist thrombin and the C-kinase activator PMA potentiated the stimulatory action of Ca^{2+} on AA release, producing a left-shift in the Ca^{2+} dose-response curve (Fig. 1). The mean ($\pm$ S.E.M) ED_{50} values for Ca^{2+} -stimulated AA release were 1.9 ± 0.3 μM for control samples, 0.79 ± 0.15 mM in the presence of thrombin ($P < 0.001$ versus control), and 1.2 ± 0.2 μM in PMA-treated platelets ($P < 0.01$ versus both control and thrombin) in four experiments. Maximal effects were seen at 2–5 units of thrombin/ml and 30–100 nM-PMA (not shown). All of the other C-kinase activators we have tested, i.e. the phorbol ester 4 β -phorbol 12,13-dibutyrate, the diterpene compound mezerein, and the synthetic diacylglycerol OAG, likewise enhanced platelet AA release in response to Ca^{2+} , whereas 4 α -PMA (which does not activate C-kinase) was without effect (Table 1). Submaximal concentrations of thrombin plus PMA in combination caused an additive enhancement of AA release: 0.5 units of thrombin/ml gave a 2.6-fold enhancement of 3 μM - Ca^{2+} -stimulated AA release, 10 nM-PMA caused a 2.4-fold enhancement, and the combination of thrombin and PMA caused a 4.8-fold enhancement of Ca^{2+} -stimulated AA release. This result is in sharp contrast to previous experiments in non- Ca^{2+} -depleted platelets in which PMA was shown to inhibit a variety of thrombin-stimulated events; these included phosphoinositide hydrolysis, inositol 1,4,5-trisphosphate formation, Ca^{2+} mobilization, exocytosis and AA release, with Ca^{2+} mobilization being particularly sensitive to inhibition [10,22–24]. The inhibition of agonist-stimulated responses by PMA may reflect a feedback inhibitory pathway mediated by C-kinase [22–24]. Since a further enhancement (rather than inhibition) of thrombin-stimulated AA release by PMA is seen in the present study under conditions where cytoplasmic [Ca^{2+}] is directly controlled, we suggest that the inhibition of AA release by PMA in thrombin-activated platelets results from inhibition of the Ca^{2+} mobilization required for AA release.

Liberation of AA in thrombin-stimulated platelets is

Table 2. Effects of thrombin and PMA on [^3H]arachidonyl phospholipids

Ca^{2+} -depleted platelets were exposed to Ca^{2+} at the indicated concentrations in the absence (control) or presence of thrombin (2 units/ml) or PMA (100 nM). Levels of [^3H]phosphatidylcholine and [^3H]phosphatidylinositol were measured as described in the Materials and methods section. Values are means $\pm$ S.E.M. from four to six preparations. * $P < 0.05$, ** $P < 0.01$, *** $P < 0.001$ versus control (pCa 5.5).

	Radioactivity (% of control at pCa 7)	
	pCa 7	pCa 5.5
Phosphatidylcholine		
Control	100	87.9 ± 5.5
Thrombin	96.6 ± 2.1	$59.6 \pm 2.6^{***}$
PMA	97.5 ± 3.7	$66.7 \pm 8.3^{**}$
Phosphatidylinositol		
Control	100	87.8 ± 7.2
Thrombin	86.5 ± 6.1	$44.0 \pm 6.4^*$
PMA	91.3 ± 4.9	$62.3 \pm 10.0^*$

largely accounted for by phospholipase A_2 activity [25–28], although an additional pathway for AA release catalysed by phospholipase C and diacylglycerol lipase has been proposed [29]. Both phosphatidylinositol and phosphatidylcholine are substrates for phospholipase A_2 [25,30] whereas phosphatidylcholine is not a substrate for phospholipase C [31]. In Ca^{2+} -depleted platelets, release of [^3H]AA was accompanied by a loss of radioactivity from phosphatidylcholine and phosphatidylinositol; thrombin and PMA significantly enhanced the Ca^{2+} -stimulated disappearance of these [^3H]phospholipids (Table 2). Thus, our results suggest that phospholipase A_2 can be directly activated by Ca^{2+} in whole platelets and that thrombin and PMA potentiate Ca^{2+} -stimulated phospholipase A_2 activity.

Since both thrombin and C-kinase activators enhance Ca^{2+} -stimulated AA release, we wished to explore the possibility that the effect of thrombin is in fact mediated by C-kinase. Activation of C-kinase in response to thrombin is thought to be caused by formation of the second messenger, 1,2-diacylglycerol [7]. 1,2-Diacylglycerol is phosphorylated by diacylglycerol kinase in human platelets [32] thus terminating the signal for activation of C-kinase. The diacylglycerol kinase inhibitor R59022 has been shown to potentiate diacylglycerol accumulation and C-kinase activity in thrombin-stimulated human platelets [33]. As shown in Fig. 2, the potentiation of AA release by thrombin was further increased in the presence of R59022 at a concentration (10 μM) that did not significantly affect AA release in the absence of thrombin. Loss of [^3H]phosphatidylcholine was similarly enhanced by R59022 under the same incubation conditions (not shown), implying that the phospholipase A_2 pathway is the source for the additional [^3H]AA released. Conversely, the C-kinase inhibitor H-7 [34] blunted the potentiation of Ca^{2+} -stimulated AA release due to thrombin (Fig. 2). Taken together, these results suggest that thrombin can enhance Ca^{2+} -dependent phospholipase A_2 activity in platelets through a mechanism involving C-kinase.

Fig. 2. Effects of R59022 and H-7 on thrombin-potentiated $[^3\text{H}]$ AA release

Ca^{2+} -depleted platelets were stimulated with $1\ \mu\text{M}$ free Ca^{2+} plus thrombin at the indicated concentrations in the absence (control) or presence of R59022 ($10\ \mu\text{M}$) or H-7 ($200\ \mu\text{M}$) added 5 min prior to thrombin and Ca^{2+} . Incubations were terminated after 10 min and $[^3\text{H}]$ AA release determined. Results are means $\pm$ S.E.M. of three experiments, each performed in triplicate. With the exception of the zero-[thrombin] points, the effects of R59022 and H-7 were significantly different from control ($P < 0.05$ by paired t test) at each thrombin concentration shown. R59022 and H-7 did not significantly affect the basal (no added Ca^{2+}) level of $[^3\text{H}]$ AA in the medium (4600 ± 386 c.p.m.) which was subtracted from all values shown in the Figure. ●, Control; □, R59022; ◇, H-7.

The results obtained in this study argue in favour of a role for Ca^{2+} as the link between the initial events that accompany stimulation of the platelet by Ca^{2+} -mobilizing agonists such as thrombin and the subsequent liberation of AA from phospholipids. Since platelet phospholipase A_2 is thought to provide the major route for the release of AA [25–28], and this enzyme has been found to require Ca^{2+} for activity [35–39], it follows that elevated cytoplasm $[\text{Ca}^{2+}]$ may elicit AA release by activating phospholipase A_2 . Other regulatory factors or pathways may also be important for the modulation of AA release [3,40]. In addition to stimulating the mobilization of intracellular Ca^{2+} , thrombin causes diacylglycerol formation and activation of C-kinase. The present results raise the possibility that C-kinase participates in the control of platelet AA liberation by enhancing the stimulatory effect of Ca^{2+} on phospholipase A_2 . Although we do not yet know the mechanism by which C-kinase activators increase AA production, it is relevant to point out that the endogenous phospholipase A_2 inhibitor lipocortin is reportedly inactivated by phosphorylation through a pathway involving C-kinase [41]. Since lipocortin has been detected in platelets [42], it is conceivable that C-kinase activators might enhance Ca^{2+} -dependent AA release by removing the inhibitory influence of endogenous lipocortin on phospholipase A_2 . C-kinase activa-

tors have now been shown to increase AA release and eicosanoid formation in a variety of cell types [43–45]. Thus, C-kinase may have a general role in regulation of Ca^{2+} -dependent phospholipase A_2 activity.

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