Ca²⁺-driven [³H]arachidonate release in electroporimeabilized human platelets shows an absolute requirement for MgATP²⁻

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Addition of micromolar Ca²⁺ to electroporimeabilized human platelets which had been pre-labelled with [³H]arachidonate causes release of ³H only when millimolar concentrations of a nucleoside triphosphate, e.g. ATP, are present in the incubation medium. Addition of millimolar Ca²⁺ in the absence of ATP, or preincubation with ATP before addition of micromolar Ca²⁺, fails to cause a significant increase in ³H release. Purine nucleotides are more effective than pyrimidine nucleotides in activating Ca²⁺-driven ³H release. This activation does not appear to involve phosphate transfer, since metabolically stable analogues of ATP, e.g. the $\beta\gamma$ -imido analogue, are effective in promoting ³H release.

INTRODUCTION

Activation of platelets by most excitatory agonists is accompanied by liberation of arachidonate from membrane phospholipids and subsequent metabolism of this polyunsaturated fatty acid via the cyclo-oxygenase and lipoxygenase pathways. Most of the arachidonate release observed in stimulated platelets results from the direct deacylation of phospholipids, e.g. phosphatidylcholine, by phospholipase A₂, although hydrolysis of 1,2-diacylglycerol by diacylglycerol lipase may make a minor contribution [1–4].

The signal-transduction mechanisms responsible for mediating agonist-induced activation of platelet phospholipase A₂ are not clearly defined. The purified enzyme is activated by Ca²⁺, although the Ca²⁺ concentrations reported to be required for maximal activation often approach the millimolar range [5–7]. In human platelets selectively permeabilized to Ca²⁺ with a bivalent ionophore, Ca²⁺-driven [³H]arachidonate release is observed on addition of micromolar Ca²⁺ [8]. However, addition of millimolar Ca²⁺ fails to induce arachidonate release from human platelet membrane preparations [9], although phospholipase A₂ has been shown to be present in such preparations [10]. Other factors may therefore be involved in control of human platelet phospholipase A₂ and hence allow observation of Ca²⁺-driven arachidonate release in intact or permeabilized cell preparations. Previous studies have indicated that Na⁺/H⁺ exchange [11], limited proteolysis [12], substrate availability [13], 1,2-diacylglycerol [8], lipocortin [14] or a specific guanine-nucleotide-binding protein [15] may modulate either Ca²⁺-dependent activation of phospholipase A₂ or Ca²⁺-driven [³H]arachidonate release.

In order to examine this problem further, we have characterized the properties of ³H release from electroporimeabilized human platelets pre-labelled with [³H]arachidonate. This preparation is permeable to small molecules up to a molecular mass of 800–1000 Da and hence allows selective manipulation of the concentration of the low-molecular-mass components of the cytosol [16]. The results obtained identify a further factor required for observation of Ca²⁺-driven arachidonate release in this cell.

MATERIALS AND METHODS

Blood was obtained from healthy drug-free volunteers and added to 0.17 vol. of acid-citrate-dextrose [17] as anticoagulant.

Platelet-rich plasma was obtained by centrifugation at 320 *g* for 20 min. Acetylsalicylate (0.2 mM) was added, and residual erythrocytes and leucocytes were removed by centrifugation at 800 *g* for 30 s. Platelets were collected by centrifugation at 680 *g* for 25 min. The pellet obtained was resuspended at a cell density of 2 × 10⁹ platelets/ml in a medium containing 145 mM-NaCl, 5 mM-KCl, 1 mM-MgCl₂, 1 mM-EGTA, 5.5 mM-glucose, 10 mM-Hepes, [³H]arachidonate (2 μ Ci/ml) and fatty-acid-free BSA (10 mg/ml) at pH 7.4. In some experiments 5-hydroxy-[¹⁴C]tryptamine (0.1 μ Ci/ml) was also added to the platelet suspension. The suspension was incubated for 60 min at 37 °C, and the labelled platelets were then collected by centrifugation at 680 *g* for 25 min. The platelets were resuspended at a cell density of (0.8–1.2) × 10⁹ platelets/ml in a medium containing 280 mM-glycine, 20 mM-potassium glutamate, 20 mM-Hepes, 4 mM-magnesium acetate and 1 mM-EGTA at pH 7.4.

The platelets were rendered permeable by ten discharges of a 20 kV · cm⁻¹ electric field [18]. Portions (20 μ l) of the permeabilized platelet preparation were diluted to 200 μ l with the glycine/glutamate medium, pH 7.4, as described above but also containing 2.5 mM-EGTA, 7.5 mM-EDTA and an appropriate concentration of Ca plus additions as specified in the Figure and Table legends. Nucleotides were added with an equivalent concentration of magnesium acetate. After incubation for 20 min at 20 °C, ice-cold 0.1 M-EGTA/20 mM-Hepes, pH 8.0, was added to stop the reaction, and the samples were immediately placed on ice. The platelets were removed by centrifugation at 8000 *g* for 2 min. Samples (50 μ l) of the cell-free supernatant containing released [³H]arachidonate + lipoxygenase metabolites were added to 4 ml of liquid scintillant (BDH cocktail T), and the ³H and ¹⁴C contents were measured in an LKB-Wallac Minibeta liquid-scintillation spectrometer.

For phospholipid analysis the reaction was stopped by addition of 3.75 vol. of chloroform/methanol (1:2, v/v). Lipids were extracted by the method of Bligh & Dyer [19]. Solvents were removed by evaporation under N₂, and the lipid fraction was redissolved in 50 μ l of chloroform. Portions (20 μ l) of this lipid extract were applied to silica-gel 60 t.l.c. plates. Phospholipids were separated by developing the plates in chloroform/propionic acid/propan-1-ol/water (10:15:10:4, by vol) and were located on the developed plate by exposure to I₂ vapour. The phospholipids were identified by their co-migration with phospholipid standards. The area of the plate corresponding to each phospho-

Abbreviations used: ATP[S], adenosine 5'-[γ -thio]triphosphate; p[NH]ppA, adenosine 5'-[$\beta\gamma$ -imido]triphosphate; p[CH₂]ppA, adenosine 5'-[$\beta\gamma$ -methylene]triphosphate.

lipid was scraped, and the ^3H content determined by liquid-scintillation spectrometry.

12-Hydroxyeicosatetraenoic acid was assayed by h.p.l.c. by using u.v. detection as described by Kanaji *et al.* [20].

Ca^{2+} concentrations were calculated by using a computer program based on ref. [21] and equilibrium constants of $10^{-7.512}$ M for CaEGTA, $10^{-2.19}$ M for MgEGTA, $10^{-7.705}$ M for CaEDTA, $10^{-5.81}$ M for MgEDTA, $10^{-3.783}$ M for CaATP and $10^{-3.92}$ M for MgATP [22]. Nucleoside triphosphates other than ATP were assumed to have equilibrium constants for their Ca and Mg complexes similar to those for ATP. The CaEDTA/EGTA buffers were prepared fresh as required by using CaCl_2 .

Unless otherwise stated, the data presented are derived from at least three replicate experiments with determinations performed in triplicate. The data points are shown as means $\pm$ S.E.M. The statistical significance of differences was assessed by Student's *t* test for unpaired comparisons.

$[^3\text{H}]$ Arachidonate (202 Ci/mmol) and 5-hydroxy $[^{14}\text{C}]$ tryptamine (57 mCi/mmol) were obtained from Amersham International, ATP[S] and p[NH]ppA from Boehringer Mannheim, and p[CH₂]ppA and all other nucleotides and phospholipids from Sigma Chemical Co. All other reagents used were analytical grade.

RESULTS

Fig. 1 demonstrates that Ca^{2+} -driven ^3H release can be

Fig. 1. Release of $[^3\text{H}]$ arachidonate and its metabolites (Δ , $\blacktriangle$) and secretion of 5-hydroxy $[^{14}\text{C}]$ tryptamine ($\circ$, $\bullet$) from electroporated human platelets measured as a function of Ca^{2+} concentration in the presence ($\bullet$, Δ) and absence ($\circ$, $\blacktriangle$) of 5 mM-MgATP $^{2-}$.

Platelets were prepared, permeabilized and incubated with the Ca^{2+} concentrations indicated, and ^3H and ^{14}C releases measured, as described in the Materials and methods section. The data shown are means $\pm$ S.E.M. of three experiments using triplicate determinations.

observed by using the electroporated human platelet preparation pre-labelled with $[^3\text{H}]$ arachidonate. The threshold Ca^{2+} concentration for observation of such release was approx. $0.1 \mu\text{M}$, and half-maximal release was obtained in the presence of $0.31 \pm 0.06 \mu\text{M}-\text{Ca}^{2+}$ ($n = 15$). These values are similar to those reported previously by Halenda & Rehm [8] in platelets selectively permeabilized to Ca^{2+} with a bivalent-cation ionophore. However, in contrast with that report, Ca^{2+} -driven ^3H release from electroporated human platelets was only observed if millimolar concentrations of MgATP $^{2-}$ were added to the incubation medium (Fig. 1). Under these conditions Ca^{2+} -driven ^3H release was linear for at least 20 min at 20°C . In order to observe this effect, MgATP $^{2-}$ had to be present in the incubation buffer. Prior incubation of electroporated platelets with 5 mM-MgATP $^{2-}$ in the presence of less than $1 \text{ nM}-\text{Ca}^{2+}$, followed by dilution into a buffer containing $5 \mu\text{M}-\text{Ca}^{2+}$ but no MgATP $^{2-}$, gave no Ca^{2+} -dependent ^3H release (results not shown). Analysis of the ^3H content of the major phospholipid classes under similar conditions showed a selective Ca^{2+} - and MgATP $^{2-}$ -dependent decrease in the ^3H content of phosphatidylcholine, with no statistically significant change in the ^3H content of the other phospholipids examined (Table 1). Under the conditions used, the ^3H released was recovered primarily in lipoxygenase metabolites, together with a small amount of arachidonate.

The conditions required to observe Ca^{2+} -driven ^3H release from this preparation are similar to those under which Ca^{2+} -driven secretion is obtained [23]. It was therefore important to determine whether the observed ^3H release was a consequence of secretion. In the studies shown in Fig. 1, the platelets had also been preincubated with 5-hydroxy $[^{14}\text{C}]$ tryptamine to label the amine-storage granules. Fig. 1 shows that the dose/response curve for Ca^{2+} -driven ^{14}C release in the presence of MgATP $^{2-}$ lay clearly to the right of that for Ca^{2+} -driven ^3H release. Half-maximal ^{14}C release under these conditions (pH 7.4) was obtained at $0.8 \pm 0.1 \mu\text{M}-\text{Ca}^{2+}$. Similar results to those shown in Fig. 1 were obtained in studies performed at pH 6.6 in a CaEGTA buffer, except that somewhat higher Ca^{2+} concentrations were required to induce both ^3H and ^{14}C release (results not shown).

Since in some preparations of phospholipase A_2 activation is only observed at near-millimolar concentrations of Ca^{2+} [5,6], we also examined ^3H release from platelets pre-labelled with $[^3\text{H}]$ arachidonate over a wider range of Ca^{2+} concentration. Although some increase in ^3H release in the absence of MgATP $^{2-}$ was observed as $[\text{Ca}^{2+}]$ was increased to the millimolar range, stimulation of release by MgATP $^{2-}$ was still obtained. The extent of stimulation declined as $[\text{Ca}^{2+}]$ was increased, due both to the increased release in the absence of MgATP $^{2-}$ and also to a decline in ^3H release obtained in the presence of MgATP $^{2-}$ at higher $[\text{Ca}^{2+}]$ (Fig. 2).

Table 1. Ca^{2+} - and MgATP $^{2-}$ -induced changes in the ^3H content of the major phospholipid classes in platelets labelled with $[^3\text{H}]$ arachidonate

Platelets were prepared, labelled with $[^3\text{H}]$ arachidonate, permeabilized and incubated with the Ca^{2+} concentration as indicated in the absence or presence of 5 mM-MgATP $^{2-}$ as described in the Materials and methods section. Phospholipids were extracted and analysed as described in the Materials and methods section. The data shown are means $\pm$ S.E.M. of results from four different experiments using triplicate determinations: * $P < 0.05$.

Phospholipid	^3H content (c.p.m.)			
	- MgATP $^{2-}$		+ MgATP $^{2-}$	
	< 1 nM- Ca^{2+}	$7 \mu\text{M}-\text{Ca}^{2+}$	< 1 nM- Ca^{2+}	$7 \mu\text{M}-\text{Ca}^{2+}$
Phosphatidylcholine	1494 ± 195	1603 ± 128	1600 ± 158	$1070 \pm 159^*$
Phosphatidylethanolamine	912 ± 70	788 ± 44	864 ± 56	716 ± 69
Phosphatidylserine	456 ± 47	378 ± 41	475 ± 46	362 ± 45
Phosphatidylinositol	352 ± 53	334 ± 62	295 ± 52	300 ± 73

Fig. 2. Release of [³H]arachidonate and its metabolites in the absence (●) or presence (○) of 5 mM-MgATP²⁻ measured over a wide range of Ca²⁺ concentrations

Platelets were prepared, permeabilized and incubated with the Ca²⁺ concentrations indicated, and ³H release was measured, as described in the Materials and methods section. The data shown are means $\pm$ S.E.M. of four experiments using triplicate determinations.

Fig. 3. Dependence of Ca²⁺-driven release of [³H]arachidonate and metabolites on MgATP²⁻ concentration

Platelets were prepared, permeabilized and incubated with the MgATP²⁻ concentrations as indicated in a system containing a fixed total Ca²⁺ content (7.9 mM), and ³H release was measured as described in the Materials and methods section. The calculated [Ca²⁺] ranged from 5.5 to 6.7 μ M over the range of MgATP²⁻ concentrations used. The data shown are means $\pm$ S.E.M. of four experiments using triplicate determinations.

At a near constant and saturating concentration of Ca²⁺, the extent of ³H release was a function of MgATP²⁻ concentration in the millimolar range (Fig. 3). Since release of ³H does not saturate over the range tested, the concentration of MgATP²⁻ required to support half-maximal release cannot be clearly defined. However, the results shown in Fig. 3 indicates a value in excess of 5 mM.

We have examined the nucleotide specificity for this effect using both other nucleoside triphosphates and also metabolically stable analogues of ATP. The results of these studies, which were performed at a fixed nucleotide concentration (5 mM) and two fixed Ca²⁺ concentrations, are summarized in Table 2. In the absence of added Ca²⁺ (estimated [Ca²⁺] $\leq$ 1 nM), no significant increase in ³H release occurred on addition of any of the nucleotides tested, in accord with the results shown for ATP in Figs. 1 and 2. In the presence of 1.7 μ M-Ca²⁺, which supports near-maximal release induced by MgATP²⁻ (Fig. 1), all the nucleotides tested except AMP and UTP induced significant ³H release. The rank order of potency under these conditions was ATP = ADP > ITP = GTP > CTP > UTP = 0. Significant ³H release also occurred in the presence of all three metabolically stable ATP analogues tested, although ATP[S] was significantly more effective than either p[NH]ppA or p[CH₂]ppA (Table 2).

Table 2. Nucleotide specificity for activation of the release of [³H]arachidonate + metabolites induced by addition of 1.7 μ M-Ca²⁺

Platelets were prepared, permeabilized, incubated with the nucleotides (5 mM) and inhibitors as indicated either in the absence of added Ca²⁺ ([Ca²⁺] $\leq$ 1 nM) or in the presence of 1.7 μ M-Ca²⁺, and ³H release was measured, as described in the Materials and methods section. The inhibitor concentrations used were 100 μ M-BW755C and 50 μ M-nordihydroguaiaretic acid (NDGA). The data shown are means $\pm$ S.E.M. of at least three experiments. The extent of Ca²⁺-induced ³H release in the presence of the specified nucleotide was compared with that in the control by Student's *t* test for unpaired data. For the inhibitors, comparison was made with Ca²⁺-dependent release in the presence of ATP but in the absence of inhibitor (**P* < 0.01, ***P* < 0.001).

Nucleotide	³ H release (c.p.m.) at:	
	< 1 nM-Ca ²⁺	1.7 μ M-Ca ²⁺
None	143 $\pm$ 16	177 $\pm$ 14
AMP	150 $\pm$ 13	189 $\pm$ 8
ADP	126 $\pm$ 12	288 $\pm$ 26**
ATP	132 $\pm$ 13	290 $\pm$ 23**
ATP[S]	139 $\pm$ 16	351 $\pm$ 28**
p[NH]ppA	152 $\pm$ 15	237 $\pm$ 23*
p[CH ₂]ppA	140 $\pm$ 13	215 $\pm$ 12*
GTP	134 $\pm$ 8	249 $\pm$ 20**
ITP	138 $\pm$ 11	262 $\pm$ 11**
CTP	134 $\pm$ 12	228 $\pm$ 22*
UTP	132 $\pm$ 8	201 $\pm$ 20
ATP + BW755C	136 $\pm$ 11	246 $\pm$ 23*
ATP + NDGA	120 $\pm$ 10	206 $\pm$ 11**

DISCUSSION

The data presented here provide an explanation for the apparent discrepancy in the results obtained when Ca²⁺-driven [³H]arachidonate release is studied in permeabilized human platelets [8] and in human platelet membrane fractions [9]. The failure to observe significant Ca²⁺-driven release in the latter system is clearly attributable to the requirement for addition of a nucleoside triphosphate, as revealed by our studies using the electroporeabilized preparation (Fig. 1 and Table 2). Such a requirement would not have been detected in platelets selectively permeabilized to Ca²⁺ with a bivalent-cation ionophore, since this preparation does not release nucleotides [8]. In similar studies on saponin-permeabilized platelets, MgATP²⁻ was present in the incubation medium [15]. It is likely that ADP is effective in this system (Table 2), owing to its conversion into ATP, as has previously been proposed for amine-storage granule and lysosomal secretion [22]. It is, however, clear that ³H release is not dependent on amine-storage-granule secretion, since the latter process occurs over a higher range of Ca²⁺ concentration (Fig. 1).

The mechanism by which nucleoside triphosphates stimulate Ca²⁺-driven ³H release is unclear, but seems unlikely to involve phosphate transfer either to phospholipase A₂ or to a protein which regulates this enzyme. This conclusion is based on two findings. First, MgATP²⁻ is effective only when added together with Ca²⁺ (Fig. 1). No ³H release is obtained if the permeabilized platelets are preincubated with MgATP²⁻ before addition of Ca²⁺. Second, the Mg complexes of ATP analogues which are unable to transfer a phosphate group, e.g. p[NH]ppA and p[CH₂]ppA, do support Ca²⁺-driven ³H release, albeit somewhat less effectively than MgATP²⁻ (Table 2). It is also improbable that addition of MgATP²⁻ permits observation of Ca²⁺-driven

[³H]arachidonate release either by enhancing the labelling of the phospholipids which are degraded by phospholipase A₂ or by influencing the rate of arachidonate utilization. The former possibility is not consistent with the protocol used, in which intact platelets are preincubated with [³H]arachidonate and then washed before permeabilization and exposure to Ca²⁺, or with the finding that MgATP²⁻ is only effective when added together with Ca²⁺. An effect on arachidonate utilization can be excluded for the cyclo-oxygenase pathway, since the studies described here were all performed in aspirin-treated preparations. An effect on the lipoxygenase pathway is also unlikely, since the extent of Ca²⁺-driven ³H release in the presence of MgATP²⁻ is not markedly decreased by addition of compounds, e.g. BW755C, nordihydroguaiaretic acid (Table 2), which have been reported to inhibit 12-lipoxygenase [24]. We have confirmed efficacy on platelet 12-lipoxygenase by demonstrating that these inhibitors markedly decrease the extent of 12-hydroxyicosatetraenoic acid formation induced by stimulation with thrombin or exogenous arachidonate (results not shown).

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