Arachidonic acid-induced calcium influx in human platelets

Comparison with the effect of thrombin

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The effects of arachidonic acid and thrombin on calcium movements have been studied in fura-2-loaded platelets by a procedure which allows simultaneous monitoring of the uptake of manganese, a calcium surrogate for Ca^{2+} channels, and the release of Ca^{2+} from intracellular stores. Arachidonic acid induced both Ca^{2+} (Mn^{2+}) entry through the plasma membrane and Ca^{2+} release from the intracellular stores. The release of Ca^{2+} was prevented by cyclo-oxygenase inhibitors and mimicked by the prostaglandin H_2 /thromboxane A_2 receptor agonist U46619. Ca^{2+} (Mn^{2+}) entry required higher concentrations of arachidonic acid and was not prevented by either cyclo-oxygenase or lipoxygenase inhibitors. Several polyunsaturated fatty acids reproduced the effect of arachidonic acid on Ca^{2+} (Mn^{2+}) entry, but higher concentrations were required. The effects of maximal concentrations of arachidonic acid and thrombin on the uptake of Mn^{2+} were not additive. Both agonists induced the entry of Ca^{2+} , Mn^{2+} , Co^{2+} and Ba^{2+} , but not Ni^{2+} , which, in addition, blocked the entry of the other divalent cations. However, arachidonic acid, but not thrombin, increased a Ni^{2+} -sensitive permeability to Mg^{2+} . The effect of thrombin but not that of arachidonic acid was prevented either by pretreatment with phorbol ester or by an increase in cyclic-AMP levels. Arachidonic acid also accelerated the uptake of Mn^{2+} by human neutrophils, rat thymocytes and Ehrlich ascites-tumour cells.

INTRODUCTION

Stimulation of platelets by a variety of agonists produces a rise in the cytoplasmic free Ca^{2+} concentration ($[\text{Ca}^{2+}]_i$) due to both release of Ca^{2+} from the intracellular stores and entry through the plasma membrane [1–3]. The nature and the activation mechanism of the entry pathway are little known [4]. Ca^{2+} channels have been recently incorporated into lipid planar bilayers using plasma membranes prepared from thrombin-stimulated platelets; these channels were also permeable to Mn^{2+} and were blocked by Ni^{2+} [5]. This is consistent with previous reports showing that thrombin induced a Ni^{2+} -inhibitable entry of Ca^{2+} or Mn^{2+} into quin2-loaded cells [6]. These observations have recently been extended to other agonists using fura-2-loaded platelets [7–10]. The spectral properties of fura-2 allow, in addition, the separate quantification of the entry of Mn^{2+} through the plasma membrane and the release of Ca^{2+} from intracellular stores, both of which take place simultaneously with most agonists [9,10].

Externally added arachidonic acid is able to activate human platelets. A biphasic effect is observed, with low ($< 20 \mu\text{M}$) concentrations inducing aggregation and secretion which are sensitive to aspirin, and larger concentrations producing aspirin-insensitive activation [11–14]. We have reported previously that high concentrations of arachidonic acid accelerate the entry of Mn^{2+} into human platelets and that this effect is blocked by Ni^{2+} [10]. It has also been shown that arachidonic acid induces ^{45}Ca release from plasma membrane vesicles [15] and from microsomes [16] prepared from human platelets.

Thrombin induces release of arachidonic acid from membrane phospholipids by activation of phospholipase A_2 [17]. It has been reported recently that Ca^{2+} mobilization induced by thrombin is decreased by treatment of platelets with the phospholipase A_2 inhibitor ONO-RS-082 [15]. On the other hand, microinjection

of phospholipase A_2 increased Ca^{2+} influx in cultured endothelial cells [18]. These results suggest the interesting possibility that arachidonic acid might mediate the effects of thrombin on Ca^{2+} mobilization. In this paper, the effects of arachidonic acid on Ca^{2+} mobilization in human platelets are analysed in detail and compared with the effects of thrombin.

EXPERIMENTAL

Platelets were prepared from freshly drawn human blood as described elsewhere [19,20] and suspended at 10^9 cells/ml in an incubation medium containing (in mM): NaCl, 140; KCl, 5; MgSO_4 , 1; glucose, 10; and sodium-Hepes, 10 (pH 7.4). Apyrase ($20 \mu\text{g}/\text{ml}$) was added to prevent activation by traces of ADP. The cells were loaded with fura-2 by incubation with $2\text{--}5 \mu\text{M}$ -fura-2/AM for 45 min at room temperature. The platelet suspension was then diluted three times with incubation medium containing 2% acid citrate-dextrose solution (ACD), and the cells were sedimented by centrifugation ($350 g$, 20 min) and resuspended at $2 \times 10^8/\text{ml}$ in fresh incubation medium.

Fluorescence was measured at 37°C and under magnetic stirring in 0.5 ml portions of cell suspension using a fluorescence spectrophotometer constructed by Cairn Research Ltd. (Newnham, Sittingbourne, Kent, U.K.). The system allows quick (30–300 Hz) alternation of up to six excitation wavelengths and separate reading of the corresponding fluorescence emissions. In the present experiments fluorescence was excited at 340, 360, 380, 450 and 492 nm and read at 530 nm. Readings were integrated at 1 s intervals.

Activation of plasma membrane Ca^{2+} channels and release of Ca^{2+} from intracellular stores were monitored simultaneously and independently in fura-2-loaded platelets by using the procedure described elsewhere [9,10]. Briefly, Mn^{2+} was added to the extracellular medium, which was nominally Ca^{2+} -free, to act as a

Abbreviations used: $[\text{Ca}^{2+}]_i$, cytoplasmic free calcium concentration; IBMX, isobutylmethylxanthine; TPA, 12-*O*-tetradecanoylphorbol 13-acetate; NDGA, nordihydroguaiaretic acid; PKC, protein kinase C; BCECF, 2',7'-bis-(2-carboxyethyl)-5(6)-carboxyfluorescein; BW755C, 3-amino-1-[*m*-(trifluoromethyl)phenyl]-2-pyrazoline; TPEN, tetrakis(2-pyridylmethyl)ethylenediamine; TMA, trimethylamine; HETE, hydroxyicosatetraenoic acid; HHT, hydroxyheptadecatrienoic acid; PAF, platelet-activating factor; ACD, acid citrate-dextrose.

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Ca^{2+} surrogate for Ca^{2+} channels. The uptake of Mn^{2+} was monitored by the quenching of fura-2 fluorescence, which can be estimated either from the decrease of the fluorescence excited at 360 nm (F_{360}), a wavelength not sensitive to changes in $[\text{Ca}^{2+}]$ [21], or from the decrease of ' F_{total} ', this being the sum of the corrected F_{340} and F_{380} values [9,10]. Both kinds of measurements gave identical results. $[\text{Ca}^{2+}]_i$ was calculated from the ratio F_{340}/F_{380} [21], which is not modified by Mn^{2+} quenching as long as the total Mn^{2+} concentration does not exceed that of fura-2. Under the conditions of the experiments, with no external Ca^{2+} , the increase in $[\text{Ca}^{2+}]_i$ reflects only the release of Ca^{2+} from the intracellular stores.

The uptake of other divalent cations was also monitored by changes of fura-2 fluorescence. Co^{2+} and Ni^{2+} quenched the fluorescence of fura-2 at any excitation wavelength. Their uptake can then be monitored in the same way as for Mn^{2+} (see above). Ba^{2+} increased F_{340} and F_{380} and decreased F_{360} . Its uptake can thus be monitored independently of changes of $[\text{Ca}^{2+}]_i$ by the increase in F_{360} . Measured values of K_d , estimated by displacement of Ca^{2+} from fura-2 and assuming for this cation a K_d of 220 nM [21] were (in nM): Mn, 1.6; Co, 3.2; Ni, 10; Ba, 1000.

Intracellular pH (pH_i) was measured in platelets previously loaded with bis-carboxyethylcarboxyfluorescein (BCECF [22]) by incubation with 0.5–2 μM of the acetoxymethyl ester under conditions similar to those described above for fura-2. In some experiments the cells were loaded simultaneously with both fura-2 and BCECF. Intracellular pH was estimated from the ratio of the fluorescences excited at 492 and 450 nm and measured at 530 nm in the Cairn spectrofluorimeter described above. Calibrations were performed with nigericin in high- K^+ medium [23].

Mg^{2+} fluxes were measured in platelets first loaded with the Mg^{2+} -sensitive dye mag-indo-1 [24] by incubation with 8 μM of the acetoxymethyl ester under conditions similar to those described above for fura-2. Loaded cells were then incubated at

37 °C under magnetic stirring in the sample compartment of a Hitachi 650-10 S spectrofluorimeter and fluorescence was measured at 340 nm excitation 410 nm emission.

Arachidonic acid metabolism was measured in cells first labelled by incubation with [^3H]arachidonic acid (30 $\mu\text{Ci}/\text{ml}$; specific radioactivity 240 Ci/mmol) in plasma containing 50 nM-prostaglandin E_1 and 20 μg of apyrase/ml for 90 min at 37 °C. The cells were then washed twice with plasma and once more with incubation medium containing 2% ACD, apyrase (20 $\mu\text{g}/\text{ml}$) and albumin (1 mg/ml). Finally the cells were resuspended at 1×10^8 cells/ml in regular incubation medium. After different periods of incubation at 37 °C with or without agonists or inhibitors the incubation was terminated by mixing 0.5 ml portions of cell suspension with 0.5 ml of ice-cold 0.5 M-formic acid. The samples were extracted with 2 vol. of ethyl acetate, and arachidonic acid metabolites were separated by t.l.c. [25].

Preparation and handling of human neutrophils [26], Ehrlich ascites-tumour cells [27] and rat thymocytes [28] were as described elsewhere.

[^3H]Arachidonic acid was obtained from Amersham International, Little Chalfont, Bucks., U.K. Free acid and acetoxymethyl ester forms of fura-2, BCECF and mag-indo-1, TPEN and 4-bromo-A23187 were from Molecular Probes, Eugene, OR, U.S.A. Thrombin (human) was from Calbiochem-Behring, La Jolla, CA, U.S.A. U46619 and BW755C were generously provided by Dr. Pilar Ortega, Laboratorios Alter, Madrid, Spain and Dr. S. Moncada, The Wellcome Research Laboratories, Beckenham, Kent, U.K., respectively. Apyrase, fatty acids and inhibitors were from Sigma Chemical Co., Poole, Dorset, U.K.

RESULTS

Fig. 1 compares the effects of several concentrations of arachidonic acid on the uptake of Mn^{2+} (Fig. 1a) and on the

Fig. 1. Concentration-dependence of the effects of arachidonic acid on the uptake of Mn^{2+} and on the release of Ca^{2+} from the intracellular stores in human platelets loaded with fura-2

(a, b) The experiments were started by the addition of MnCl_2 to give a final concentration of 0.2 mM in the cell suspension, followed 1 min later by various concentrations of arachidonic acid ($\bullet$, 10 μM ; $\circ$, 20 μM ; $\triangle$, 30 μM ; $\blacktriangle$, 90 μM). Total fluorescence was calculated as $F_{340} + k \times F_{380}$, where k represents $[(F_{\text{max}} - F_{\text{min}})_{340}]/[(F_{\text{max}} - F_{\text{min}})_{380}]$. F_{max} and F_{min} were the values of fluorescence with excess Ca^{2+} or excess EGTA respectively obtained after lysis of the cell suspension with Triton X-100 [9,10]. With our particular set of filters the value of k was 0.62. $[\text{Ca}^{2+}]_i$ was calculated from the ratio F_{340}/F_{380} [21]. In all the cases the values of fluorescence were corrected by subtraction of the autofluorescence, obtained by quenching fura-2 fluorescence of the lysed cell suspension by excess Mn^{2+} . (c) Results expressed as percentages of the maximal effect. The entry of Mn^{2+} ($\circ$) was calculated as the maximal rate of quenching of the total fluorescence obtained during the first 15 s. The increase in $[\text{Ca}^{2+}]_i$ ($\bullet$) was calculated from the maximum value obtained at the peak of the trace. The results shown are representative of seven similar experiments performed with different blood samples.

release of Ca^{2+} from the intracellular stores (Fig. 1*b*). Arachidonic acid produced both a concentration-dependent increase of Mn^{2+} uptake and a release of Ca^{2+} from the intracellular stores, but the latter effect was decreased by increasing the concentration of arachidonic acid above $10\text{ }\mu\text{M}$ (Fig. 1*b*). Fig. 1*c* shows a dose-response curve summarizing the results of several similar experiments. A bell-shaped curve centred near $10\text{ }\mu\text{M}$ -arachidonic acid was observed for Ca^{2+} release. This is consistent with previous reports [13,14]. In contrast, the effect on Mn^{2+} uptake appeared only above $10\text{ }\mu\text{M}$ and was maximal (estimated rate near 70 mmol/h per litre of cells) at $60\text{ }\mu\text{M}$.

Since the concentrations of arachidonic acid needed to increase the uptake of Mn^{2+} were rather high, it was critical to establish that these effects were not due to non-selective permeabilization of the plasma membrane. None of the concentrations of arachidonic acid used resulted in release of either lactate dehydrogenase or fura-2 into the incubation medium (results not shown). It could be argued, however, that the membrane permeability to smaller molecules could be increased. Fig. 2 shows that the quenching of fura-2 fluorescence induced by addition of arachidonic acid in Mn^{2+} -containing medium was prevented by the simultaneous presence of Ni^{2+} . It can be seen that the subsequent addition of ionomycin, which does not transport Ni^{2+} , induced quenching of fura-2 by Mn^{2+} entering. The third trace in Fig. 2 shows that arachidonic acid did not induce uptake of Ni^{2+} , since no quenching of fura-2 fluorescence was produced. The subsequent addition of alamethicin, a divalent cation ionophore with little specificity [29], resulted in entry of Ni^{2+} . These results show that: (i) arachidonic acid treatment does not release fura-2 into the medium; (ii) it does not increase the plasma membrane permeability to Ni^{2+} ; and (iii) Ni^{2+} prevents the increase in the uptake of Mn^{2+} induced by arachidonic acid. Ni^{2+} did not modify the release of Ca^{2+} from the intracellular stores induced by arachidonic acid (results not shown).

Fig. 3 shows the effects of the sequential addition of Mn^{2+} , EGTA and TPEN on the fluorescence of fura-2-loaded platelets. After addition of Mn^{2+} there was a quick decrease in fluorescence which was due to quenching of the extracellular dye leaked out from the cells. This was followed by a slower decrease due to entry of Mn^{2+} . On addition of EGTA, which chelates extracellular divalent cations, there was an abrupt increase in fluorescence. This corresponds to the increase in the fluorescence of extracellular fura-2 on going from the quenched state (fura-2- Mn^{2+} complex) to the minimal fluorescence state (unbound

Fig. 2. Effects of Ni^{2+} on the uptake of Mn^{2+} induced by arachidonic acid in fura-2-loaded platelets

The incubation medium contained either 0.2 mM-Mn^{2+} , 0.2 mM-Mn^{2+} plus 5 mM-Ni^{2+} or 5 mM-Ni^{2+} . Additions of arachidonic acid (AA, $60\text{ }\mu\text{M}$), ionomycin (Iono, $0.2\text{ }\mu\text{M}$) or alamethicin (ALA, $50\text{ }\mu\text{g/ml}$) are marked. Other details were as in the legend to Fig. 1.

Fig. 3. Effects of EGTA and TPEN on the Mn^{2+} content of fura-2-loaded platelets treated with arachidonic acid

In the first trace the effects on cells not treated with arachidonic acid were shown as a control. Additions, as shown by the arrows, were at the following concentrations (in mM): Mn^{2+} , 0.2 ; EGTA, 5 ; TPEN, 0.03 (added from a concentrated stock in dimethyl sulphoxide); arachidonic acid (AA), 0.06 . Other details were as in the legend to Fig. 1.

fura-2). The fluorescence then remains stable, indicating that Mn^{2+} is no longer entering the cells. Addition of TPEN, a lipophilic membrane-permeable chelator which binds heavy divalent cations but not Ca^{2+} or Mg^{2+} [30], had little or no effect. In the right-hand trace (Fig. 3), the addition of arachidonic acid after Mn^{2+} greatly accelerated the quenching of fura-2 fluorescence, reflecting Mn^{2+} uptake. The subsequent addition of EGTA produced an abrupt rise in fluorescence followed by a slower rise. This suggests a slow exit of Mn^{2+} on chelation of the extracellular cation. The addition of TPEN then produced a fast increase in fluorescence, reflecting the binding of intracellular Mn^{2+} by the entering lipophilic chelator. These results prove that the cell membrane is still acting as a barrier for fura-2, EGTA and Mn^{2+} after treatment with arachidonic acid.

Additional support for the selectivity of the action of arachidonic acid was obtained by studying its effects on H^{+} movements. Fig. 4 shows the results of a typical experiment with platelets loaded with BCECF. Mn^{2+} was added to the medium in order to simulate exactly the same conditions as in the Mn^{2+} -influx experiments. In the first trace the addition of the weak base

Fig. 4. Effects of the addition of TMA on intracellular pH of control and arachidonic acid-treated BCECF-loaded platelets

Additions as shown by arrows were at the following concentrations: Mn^{2+} , 0.2 mM ; TMA, 10 mM ; Triton X-100 (TX), 0.05% ; arachidonic acid (AA), $60\text{ }\mu\text{M}$. Downwards deflections indicate intracellular acidification. The value of pH_i at the start of the experiment was about 7.30 . The alkalinization by TMA is equivalent to about 0.2 pH units.

trimethylamine (TMA) produced an increase in fluorescence. This reflects the faster uptake of the uncharged species relative to the trimethylammonium ion, resulting in cytoplasmic alkalization [31]. The detergent Triton X-100 was added about 30 s later, resulting in a large increase in fluorescence. This arises mostly from the shift of the BCECF spectrum on release from the cells [32]. As shown in the second trace, the addition of arachidonic acid produced a quick decrease of fluorescence (acidification) followed by a slower increase which slightly surpassed the level before arachidonic acid addition. A similar biphasic pH_i shift has been reported before in response to thrombin. The fast acidification arises from an increased production of H⁺ and the slower alkalization is the result of the stimulation of Na⁺/H⁺ exchange [20,33,34]. It seems from these results that arachidonic acid reproduced the effects of thrombin on H⁺ movements. Although this point was not addressed here, the stimulation of Na⁺/H⁺ exchange should be expected, since these concentrations of arachidonic acid have been reported to stimulate directly protein kinase C (PKC) [13,35], which could, in turn, activate Na⁺/H⁺ exchange [36]. What matters here is that addition of TMA after arachidonic acid produced the same cytoplasmic alkalization as seen in control cells which were not treated with arachidonic acid. This result proves that: (i) the physiological difference in permeability between the charged and the uncharged species of TMA was preserved; and (ii) the plasma membrane permeability to H⁺ was not changed by arachidonic acid. The final addition of Triton X-100 produced the same changes in fluorescence as in the control cells, again supporting the view that the cells were not lysed before the addition of the detergent.

Arachidonic acid is metabolized in human platelets through two main pathways. The cyclo-oxygenase pathway results in thromboxanes and prostaglandins and it can be inhibited by aspirin [17]. The lipoxygenase pathway results in hydroperoxy and hydroxy derivatives and can be blocked by nordihydroguaiaretic acid (NDGA) [17,37,38]. In order to assess whether the effects of arachidonic acid were mediated by one of these metabolites, the effects of inhibitors were tested. Fig. 5 shows that aspirin treatment blocked the release of Ca²⁺ from the intracellular stores effected by arachidonic acid. On the other hand, the prostaglandin H₂/thromboxane A₂ receptor agonist U46619 [3] reproduced the action of arachidonic acid on Ca²⁺ release, the effect being, in this case, insensitive to aspirin (Fig. 5). These results indicate that the release of Ca²⁺ from the intracellular stores induced by arachidonic acid is mediated by cyclo-oxygenase metabolites. This is consistent with previous evidence [11–13].

On the contrary, the effects of arachidonic acid on the uptake of Mn²⁺ were not mimicked by U46619 (Fig. 6) or inhibited by aspirin (Fig. 7a). Fig. 7(b) shows that treatment with NDGA also failed to modify the effects of arachidonic acid on the uptake of Mn²⁺. The dual cyclo-oxygenase and lipoxygenase inhibitor BW755C [39] also did not prevent the acceleration of Mn²⁺ uptake induced by arachidonic acid (Fig. 7c). The effects of the inhibitors on thrombin-induced arachidonic acid metabolism were tested in parallel experiments with cells labelled with [³H]arachidonic acid. Aspirin, as expected, completely prevented thromboxane A₂ formation and increased the levels of arachidonic acid, hydroxyicosatetraenoic acid (HETE) and hydroxyheptadecatrienoic acid (HHT). BW755C effectively prevented the formation of all the metabolites, i.e. thromboxane A₂, HETE and HHT, and increased the levels of free arachidonic acid. This confirms inhibition of both lipoxygenase and cyclo-oxygenase. NDGA had exactly the same effects as BW755C, suggesting that, in spite of previous claims for selective lipoxygenase inhibition [37,38], NDGA antagonizes both cyclo-

Fig. 5. Effects of aspirin on the release of Ca²⁺ from the intracellular stores by arachidonic acid and by the prostaglandin H₂/thromboxane A₂ receptor agonist U46619

After addition of fura-2/AM, the cell suspension was divided into two batches and 0.1 mM-aspirin was added to one of them (b) during the last 20 min of the loading period (a, control). NiCl₂ (5 mM) was added to the cell suspension 1 min before arachidonic acid (AA) or U46619. The same results were obtained when either 0.2 mM-MnCl₂ or 2 mM-EGTA was added instead of NiCl₂. The results are representative of three similar experiments performed with different blood samples. Other details were as in the legend to Fig. 1.

Fig. 6. Effects of successive addition of 1 μM-U46619 and 90 μM-arachidonic acid (AA) on the uptake of Mn²⁺ by human platelets

The addition of MnCl₂ (0.2 mM) was performed 1 min before the addition of U46619. Platelets had been treated with aspirin in this particular experiment, but the same results were obtained in cells not treated with aspirin. Other details were as in the legend to Fig. 1.

oxygenase and lipoxygenase. All the above results suggest that, in contrast with what is observed for Ca²⁺ release, the effects of arachidonic acid on the plasma membrane should be attributed to arachidonic acid itself and not to its metabolites.

Other fatty acids were tested in order to assess the specificity of the arachidonic acid effect. Linoleic acid reproduced the effects of arachidonic acid on the uptake of Mn²⁺, although higher concentrations were required. This fatty acid, however, was unable to induce Ca²⁺ mobilization from the intracellular stores (Fig. 8). Table 1 summarizes the effects of other fatty acids and derivatives on the uptake of Mn²⁺. At 60 μM, a concentration giving the maximal effect in the case of arachidonic acid, the

Fig. 7. Effects of aspirin (a), NDGA (b) and BW755C (c) on the uptake of Mn²⁺ induced by arachidonic acid (60 μM)

Treatment with aspirin was performed as in the legend to Fig. 5. NDGA and BW755C were added to the suspension 5 min before arachidonic acid (AA) addition (arrows) to give final concentrations of 20 and 200 μM respectively. Each experiment was performed with a different cell batch. The results are representative of three to six similar experiments. Other details were as in the legend to Fig. 1. Solid lines indicate control cells and broken lines indicate treated cells.

other fatty acids tested were at most one-twentieth as effective as arachidonic acid. At 200 μM, the following general trends were observed. (i) None of the saturated fatty acids was active; the effect required the presence of several double bonds in the *cis* configuration and increased with the degree of unsaturation. (ii) The position of the double bonds relative to the terminal methyl group was also important, the fatty acids of the ω-6 series being more active than those of the ω-3 series. (iii) A free carboxyl group was required.

Some of the fatty acids tested showed a lag of 2–5 s before the increase in Mn²⁺ uptake occurred. This is shown for linoleic acid in Fig. 8, and was also apparent for linolelaidic acid and for both eicosatrienoic acid isomers. None of the fatty acids tested, except arachidonic acid, produced noticeable Ca²⁺ release from the intracellular stores (results not shown). With some arachidonic

Fig. 8. Concentration-dependence of the effects of linoleic acid on the uptake of Mn²⁺ (a) and the release of Ca²⁺ from the intracellular stores (b)

Details were as in the legend to Fig. 1. The results shown are representative of five similar experiments with different blood samples. Concentrations of linoleic acid = ○, 30 μM; ●, 60 μM; △, 90 μM; ▲, 120 μM; □, 150 μM.

Table 1. Effects of several fatty acids and derivatives on Mn²⁺ uptake by human platelets

Fatty acid (trivial name)	Rate of Mn ²⁺ uptake (%)	
	60 μM	200 μM
C _{16:0} (palmitic)	—	3
C _{18:0} (stearic)	—	4
C _{18:1, <i>cis</i>-9} (oleic)	—	10
C _{18:1, <i>trans</i>-9} (elaidic)	—	5
C _{18:2, <i>cis</i>-9,12} (linoleic)	3	55
C _{18:2, <i>trans</i>-9,12} (linolelaidic)	—	7
C _{18:3, <i>cis</i>-9,12,15} (linolenic)	2	54
C _{18:3, <i>cis</i>-6,9,12} (γ-linolenic)	5	100
C _{18:4, <i>cis</i>-6,9,12,15} (octadecatetraenoic)	2	44
C _{20:0} (arachidic)	—	2
C _{20:2, <i>cis</i>-11,14} (eicosadienoic)	—	6
C _{20:3, <i>cis</i>-11,14,17} (eicosatrienoic)	—	16
C _{20:3, <i>cis</i>-8,11,14} (eicosatrienoic)	—	27
C _{20:4, <i>cis</i>-5,8,11,14} (eicosatetraenoic; arachidonic)	100	100
C _{20:5, <i>cis</i>-5,8,11,14,17} (eicosapentaenoic)	2	100
C _{22:1, <i>cis</i>-13} (erucic)	—	3
C _{22:4, <i>cis</i>-7,10,13,16} (docosatetraenoic)	—	30
C _{22:6, <i>cis</i>-4,7,10,13,16,19} (docosahexaenoic)	—	59
C _{24:1, <i>cis</i>-15} (nervonic)	—	3
γ-Linolenyl alcohol	—	7
Arachidonyl alcohol	—	10
Arachidonyl acetate	—	8
Arachidonic acid ethyl ester	—	8

Fig. 9. Comparison of the effects of thrombin (Δ), arachidonic acid ($\circ$) and the joint addition of both agonists ($\bullet$) on the uptake of Mn^{2+} by human platelets loaded with fura-2

The concentration of arachidonic acid was $60 \mu M$ in (a) and $6 \mu M$ (b). Thrombin was used at 1 unit/ml in all the cases. Fluorescence has been normalized to 100 at the time of addition of arachidonic acid. Note the different scales on the ordinates. Each trace is the mean of three experiments; vertical bars represent S.D. Other details were as in the legend to Fig. 1.

acid derivatives a $[Ca^{2+}]_i$ peak was observed, suggesting contamination with or fast intracellular production of some arachidonic acid.

In order to investigate whether the plasma membrane pathway opened by arachidonic acid was the same as that activated by thrombin, the additivity of the effects of the two agonists was tested. The rationale was that, if different pathways were opened by each agonist, the rate of Mn^{2+} uptake obtained after their joint addition should be the sum of the rates obtained after separate additions. The feasibility of this approach was tested by comparing the rates of Mn^{2+} uptake obtained after the joint and the separate additions of $60 \mu M$ -arachidonic acid and $0.4 \mu M$ -ionomycin. The rates obtained were (decrease of fluorescence/s; means $\pm$ S.E.M.): arachidonic acid, 9.1 ± 1.6 ; ionomycin, 18.4 ± 1.7 ; arachidonic acid+ionomycin, 28.7 ± 2.9 . Fig. 9 compares the effects of thrombin, arachidonic acid and the joint addition of both agonists on the uptake of Mn^{2+} . No additivity was observed when both agonists were used at concentrations giving maximal individual effects (Fig. 9a), suggesting that they open the same Mn^{2+} pathway. However, this interpretation was questioned by the results shown in Fig. 9(b). A low concentration of arachidonic acid, having by itself no effect on Mn^{2+} uptake was, however, able to prevent the effect of thrombin. The outcome was even more complicated when submaximal concentrations of arachidonic acid were used. In this case the effect of the joint addition of both agonists was smaller than the effect of either of them separately (results not shown).

In order to compare the selectivity of the plasma membrane

Fig. 10. Comparison of the effects of thrombin and arachidonic acid on the uptake of Ca^{2+} , Ba^{2+} , Co^{2+} and Mn^{2+} in platelets loaded with fura-2

(a) Effects on fluorescence excited at 360 nm, which is not sensitive to Ca^{2+} , but is quenched by Co^{2+} and Mn^{2+} and increased by Ba^{2+} . In the presence of Ni^{2+} (5 mM) no modifications of fluorescence were observed after the addition of arachidonic acid. These traces have been removed for simplicity. (b) Effects on the ratio of the fluorescence excited at 340 and 380 nm. This ratio increases with both Ca^{2+} and Ba^{2+} (see the Experimental section). The concentrations of divalent cations used were (in mM): Ca^{2+} , 1; Ba^{2+} , 1; Mn^{2+} , 0.2; Co^{2+} , 0.2. Thrombin (Thr) was present at 1 unit/ml and arachidonic acid (AA) was present at $60 \mu M$.

pathways opened by arachidonic acid and thrombin, the effects of these agonists on the uptake of other divalent cations were studied. Fig. 10 compares the effects on the uptake of Mn^{2+} , Co^{2+} and Ba^{2+} . Mn^{2+} and Co^{2+} quench the fluorescence of fura-2, whereas Ba^{2+} increases the fluorescence excited at 360 nm (see the Experimental section). Both agonists increased the uptake of all three divalent cations. In all cases the effects were prevented by Ni^{2+} (results not shown). The uptake of Mn^{2+} was faster than that of Co^{2+} . The rate for Ba^{2+} could not be compared directly, but the relative rates (Mn^{2+}/Ba^{2+}) seemed to be similar for arachidonic acid and for thrombin. Fig. 10(b) compares the effects of arachidonic acid and thrombin on the ratio of fluorescence excited at 340 and at 380 nm (R) in cell suspensions incubated with either Ca^{2+} or Ba^{2+} in the presence and in the absence of excess Ni^{2+} . Both agonists increased R , suggesting an increase of $[Ca^{2+}]_i$ or $[Ba^{2+}]_i$. In the presence of Ni^{2+} the increase in R was smaller and about the same for both agonists, suggesting that this increase corresponds to Ca^{2+} release from the intracellular stores. The difference with the Ni^{2+} -free condition would then represent the entry of Ca^{2+} or Ba^{2+} .

Effects on the membrane permeability to Mg^{2+} were studied in cells loaded with mag-indo-1. Fig. 11 compares the effects of the ionophore 4-bromo-A23187, arachidonic acid and thrombin in cells incubated with different Mg^{2+} concentrations. The ionophore increased the fluorescence in the cells incubated with 5 mM- Mg^{2+} , decreased it in the cells incubated with no Mg^{2+} and had little effect when the Mg^{2+} concentration was

Fig. 11. Effects of 4-bromo-A23187 (BRA, 10 μM), arachidonic acid (AA, 60 μM) and thrombin (Thr, 1 unit/ml) on the fluxes of Mg^{2+} in human platelets loaded with mag-indo-1

The crude traces of fluorescence excited at 340 nm and emitted at 410 nm are shown. Fluorescence increased with increasing $[\text{Mg}^{2+}]_i$. Cells loaded with the dye were suspended in standard incubation medium (1 mM- Mg^{2+}). Immediately before the experiment, 0.1 mM-EGTA was added to the cuvette. This was found to decrease background fluorescence, probably due to extracellular dye leaking out from the cells. Immediately before the addition of the agonist, the Mg^{2+} concentration was adjusted either to 5 mM (by addition of 4 mM- MgCl_2 , traces A), 1 mM (no additions, traces B) or 0 mM (addition of 2 mM-EDTA, traces C). The arrows mark the addition of the agonists. This experiment is representative of four similar ones performed with different blood samples. The decrease in fluorescence produced by thrombin in medium containing no Mg^{2+} was not cancelled out in the ratio of the fluorescence emissions at 410 and 485 nm.

1 mM, suggesting that this concentration approaches $[\text{Mg}^{2+}]_i$. Arachidonic acid had similar effects, suggesting that it effectively permeabilizes the cells to Mg^{2+} . The effects of arachidonic acid on Mg^{2+} fluxes were prevented by Ni^{2+} (results not shown). Thrombin produced a decrease in fluorescence with zero Mg^{2+} and did not modify it at 5 mM- Mg^{2+} . At 1 mM- Mg^{2+} thrombin produced either no effect or a small decrease in fluorescence in different experiments (results not shown). The effects of thrombin were not affected by Ni^{2+} . These results suggest that the decrease in fluorescence obtained with zero Mg^{2+} was artifactual and that thrombin does not increase plasma membrane permeability to Mg^{2+} .

In order to analyse further the similarities and the differences between the effects of arachidonic acid and thrombin, several conditions which are known to inhibit platelet function were tested. Pretreatment of platelets with phorbol esters is known to prevent the increase in $[\text{Ca}^{2+}]_i$ induced by thrombin [40–43]. Fig. 12 shows that pretreatment with 12-*O*-tetradecanoylphorbol 13-acetate (TPA) inhibited the effects of thrombin on both Mn^{2+} uptake and Ca^{2+} release from the intracellular stores. The responses to ADP and platelet-activating factor (PAF) were also fully prevented by TPA treatment (results not shown). In contrast, the effects of arachidonic acid were little affected or not at all. TPA addition *per se* did not modify the uptake of Mn^{2+} (results not shown).

Increased levels of cyclic AMP also inhibit platelet activation by several agonists [44,45]. When thrombin is used as the agonist, an increase in cyclic AMP prevents stimulation of phospholipase A_2 and, to a lesser degree, of phospholipase C [39]. Fig. 13 shows that a 2 min pretreatment with forskolin and isobutylmethylxanthine (IBMX), a treatment known to increase cyclic AMP [46], largely antagonized both the thrombin-induced release of Ca^{2+} from the intracellular stores and the acceleration of Mn^{2+} uptake. Similar effects were observed when ADP or PAF was used as the agonist (results not shown). In contrast, the

Fig. 12. Effects of pretreatment with TPA on the uptake of Mn^{2+} (a) and on the release of Ca^{2+} from the intracellular stores (b) induced by thrombin and by arachidonic acid

Cells loaded with fura-2 were suspended in standard medium containing 0.2 mM-EGTA and divided into two portions. To one portion, TPA was added to give a final concentration of 100 nM in the suspension. After 40–60 min at 25 °C the measurements were started by the addition of 0.4 mM- MnCl_2 , followed 1 min later by the addition of either thrombin (Thr, 1 unit/ml) or arachidonic acid (AA, 60 μM). Other details were as in the legend to Fig. 1. The results are representative of three similar experiments.

Fig. 13. Effects of increased cyclic AMP levels on the uptake of Mn^{2+} (a) and on the release of Ca^{2+} from the intracellular stores (b) induced by thrombin and by arachidonic acid

Cells were treated with 20 μM -forskolin + 20 μM -IBMX for 2 min before the addition of thrombin (Thr, 1 unit/ml) or arachidonic acid (AA, 60 μM). This treatment increased cyclic AMP levels from 1.4 ± 0.6 to 57.7 ± 7.5 pmol/ 10^8 cells. Other details were as in Fig. 1. The results are representative of three similar experiments.

Fig. 14. Effects of arachidonic acid on the uptake of Mn^{2+} by human neutrophils (a), rat thymocytes (b) and Ehrlich ascites-tumour cells (c)

Fura-2-loaded cells were suspended in the same incubation medium as that used for platelets. MnCl_2 was added to give a final concentration of 0.2 mM in the cell suspension, followed 1 min later by 60 μM -arachidonic acid (AA). Other details were as in Fig. 1. —, Control; ····, + 5 mM-NiCl₂.

effects of arachidonic acid on the uptake of Mn^{2+} were hardly affected by pretreatment with forskolin and IBMX.

Fig. 14 shows the effects of arachidonic acid in another three cell preparations: human neutrophils, rat thymocytes and Ehrlich ascites-tumour cells. An acceleration of Mn^{2+} uptake phenomenologically similar to that described above in platelets was found in all the cases, and this was prevented by Ni^{2+} .

DISCUSSION

We document here the effects of arachidonic acid on Ca^{2+} movements in human platelets. These include Ca^{2+} release from intracellular stores and Ca^{2+} entry through the plasma membrane. Ca^{2+} release is accepted to be mediated by InsP_3 in a large variety of systems including platelets [3,47,48]. We show here that, as suggested before [11–14], the Ca^{2+} -releasing effect of arachidonic acid is mediated by a cyclo-oxygenase metabolite, since it is prevented by aspirin and can be mimicked by the prostaglandin H_2 /thromboxane A_2 analogue U46619. A maximal effect is achieved at concentrations of about 10 μM , and larger concentrations decrease the effect. This has been attributed to the generation of lipoxygenase metabolites, which antagonize thromboxane synthesis and have general inhibitory effects [17,49].

Arachidonic acid had been proposed previously to induce entry of Ca^{2+} into platelets on the basis of the Ca^{2+} -dependence of some of its effects, and also because the increase of $[\text{Ca}^{2+}]_i$ is larger in the presence of external Ca^{2+} than in Ca^{2+} -free medium [11–14]. In the present work the use of Mn^{2+} as a surrogate for Ca^{2+} and the simultaneous and independent measurements of the plasma membrane fluxes and the mobilization from the intracellular stores have allowed a much more detailed analysis.

Since the acceleration of Mn^{2+} entry by arachidonic acid required rather high concentrations of the fatty acid, it was critical to exclude non-selective permeabilization of the membrane. We have shown that arachidonic acid does not permeabilize the membrane to Ni^{2+} , H^+ or trimethylammonium ions and that Ni^{2+} blocks the arachidonic acid-induced uptake of Mn^{2+} . The fact that none of the other fatty acids tested was able to increase the plasma membrane permeability to Mn^{2+} , except at higher concentrations (Table 1), also argues against a non-selective effect. We must conclude that the effects of arachidonic

acid on the uptake of Mn^{2+} cannot be attributed to cell lysis or to non-specific plasma membrane permeabilization.

Activation of ion channels by lipoxygenase metabolites of arachidonic acid has been reported in several cells [37,38]. In platelets neither the inhibitors of cyclo-oxygenase nor those of lipoxygenase prevented the effects of arachidonic acid on the uptake of Mn^{2+} . This suggests that arachidonic acid acts as such, with no metabolic transformation, on plasma membrane permeability. The fact that other non-metabolizable polyunsaturated fatty acids, which are unable to produce release of Ca^{2+} from the intracellular stores, also increased the plasma membrane permeability to Mn^{2+} (although only at higher concentrations) reinforces the above conclusion.

The activation of the phospholipase C/PKC system is involved in many of the effects of thrombin [1–3] and arachidonic acid and other unsaturated fatty acids are able to activate PKC directly [13,35]. Since several examples are known of channels which are regulated by phosphorylation [50–52], the possible mediation of PKC in this case should be considered. Direct activation of PKC by TPA did not, however, increase the uptake of Mn^{2+} . On the other hand, pretreatment with TPA, which should down-regulate PKC [3,53,54], did not prevent the effect of arachidonic acid. Finally, the selectivity found here for arachidonic acid over other fatty acids for acceleration of the uptake of Mn^{2+} is more strict than that reported for activation of PKC [13]. All of these observations suggest that the increase in Ca^{2+} (Mn^{2+}) influx induced by arachidonic acid is not mediated by PKC activation.

Since thrombin induces generation of arachidonic acid by activation of phospholipase A_2 [17], the possibility that arachidonic acid could mediate the effect of thrombin should be considered. It has been reported before that Ca^{2+} mobilization induced by thrombin is decreased by treatment with phospholipase A_2 inhibitor ONO-RS-082 [15]. On the other hand, microinjection of phospholipase A_2 increased Ca^{2+} influx in cultured endothelial cells [18]. The increase in cyclic AMP, which is known to block the activation of phospholipase A_2 effected by thrombin [39], prevented the acceleration of Mn^{2+} uptake induced by thrombin, but it did not modify the effect of added arachidonic acid (Fig. 13). The observation that the effects of maximal concentrations of arachidonic acid and thrombin on Mn^{2+} uptake were not additive (Fig. 9) may be interpreted to mean that both agonists

open the same Ca^{2+} (Mn^{2+}) pathway of the plasma membrane. However, the fact that low concentrations of arachidonic acid inhibited the effect of thrombin allows to reinterpret the lack of additivity as due to abolition of the effect of thrombin by high concentrations of arachidonic acid.

The divalent cation selectivity of the plasma membrane pathways opened by arachidonic acid and thrombin were similar. In addition to Ca^{2+} , the cations Ba^{2+} , Mn^{2+} and Co^{2+} , but not Ni^{2+} , could pass through this channel. Furthermore, Ni^{2+} blocked the fluxes of the other divalent cations in all cases. Arachidonic acid, but not thrombin, increased the permeability to Mg^{2+} . The Mg^{2+} fluxes were also blocked by Ni^{2+} . These results indicate that the plasma membrane pathway activated by arachidonic acid is not the same as that opened by thrombin. Consequently, it is unlikely that arachidonic acid mediates the effects of thrombin on the plasma membrane permeability to Ca^{2+} . It has been reported recently that arachidonic acid is able to release ^{45}Ca from plasma membrane vesicles [15] and from microsomes [16] prepared from human platelets. The selectivity of arachidonic acid compared with other fatty acids was similar to that reported here. These effects were attributed to a ionophoric mechanism, although the presence of an endogenous membrane component was also required [16]. Our results are consistent with this interpretation. The results obtained in the three other cell types tested here indicate that the ionophoric effects of arachidonic acid are not restricted to platelets.

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