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Arachidonic acid inhibits capacitative calcium entry in rat thymocytes and human neutrophils

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Abstract

Emptying the intracellular Ca^{2+} stores by treatment with the endomembrane Ca^{2+}-ATPase inhibitor thapsigargin activates capacitative Ca^{2+} entry (CCE). This can be evidenced in fura-2-loaded cells by an increase of $[Ca^{2+}]_i$ or by an acceleration of Mn^{2+} entry. Micromolar concentrations of arachidonic acid inhibited CCE induced by treatment with thapsigargin in rat thymocytes and in human neutrophils. This inhibitory action was shared by other unsaturated fatty acids, but not by the saturated arachidic acid nor by arachidonic acid methyl ester. The effect was not due to metabolites derived from arachidonic acid since several non-metabolizable analogs were able to reproduce it. Phorbol dibutyrate (PDB) acted similarly, suggesting that the inhibitory effect could be mediated by activation of protein kinase C (PKC). However, whereas the inhibition of CCE by PDB was reversed by treatment with the PKC inhibitor staurosporin, the inhibition by arachidonic acid was not. We find that unsaturated fatty acids antagonized microsomal dealkylation of benzyl-resorufin, a cytochrome P450-mediated activity, with the same specificity profile as for inhibition of CCE. These results are consistent with previous proposals suggesting that a microsomal cytochrome P450 may be involved in the regulation of CCE. © 1997 Elsevier Science B.V.

Keywords: Arachidonic acid; Capacitative calcium entry; Store operated Ca^{2+} entry; Cytochrome P450; Thymocyte; Neutrophil

1. Introduction

In many cell types, emptying of the intracellular calcium stores activates a plasma membrane pathway for Ca^{2+} entry known as capacitative Ca^{2+} entry (CCE, [1]), store-operated Ca^{2+} channels (SOCCs, [2]) or Ca^{2+} release-activated Ca^{2+} current (I_{CRAC}, [3]). CCE can be elicited by many physiological agonists that act primarily by releasing Ca^{2+} from the intracellular stores, thus being responsible for much of the agonist-induced Ca^{2+} entry [4]. This delayed Ca^{2+} entry secondary to emptying of the stores is essential for some of the physiological effects observed on stimulation by agonists [5]. Blockers of the endoplasmic reticulum Ca^{2+} ATPase, such as thapsigargin [6], also induce, after emptying of the stores by Ca^{2+} leak, CCE. It has been shown recently that CCE can also be down-regulated by a mechanism

Abbreviations: $[Ca^{2+}]_i$, cytosolic Ca^{2+} concentration; CCE, capacitative Ca^{2+} entry; DHA, 5,6-dehydro-arachidonic acid; ETI, 5,8,11-eicosatriynoic acid; ETYA, 5,8,11,14-eicosatetraynoic acid; AME, arachidonic acid methyl ester; PDB, phorbol 12,13-dibutyrate; PKC, protein kinase C; AA, arachidonic acid; EET, 5,6-epoxy-eicosatrienoic acid; AA-COCF3, arachidonyl trifluoromethylketone

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involving protein phosphorylation [7], which can be elicited either by several physiological agonists [8–11] or by phorbol esters [8,9].

5,6-Epoxy-eicosatrienoic acid, an oxidation product derived from arachidonic acid (AA) via P450 monooxygenases, has been proposed to activate CCE in endothelial cells [12]. AA and other unsaturated fatty acids have also been reported to promote Ca^{2+} entry in several cell types [13,14], but these fluxes are insensitive to inhibitors of CCE. On the other hand, unsaturated fatty acids have been shown to antagonize agonist-induced Ca^{2+} entry in T-lymphocytes (Jurkat line [15]) and thyroid cells (FRTL-5 line [16]). Unsaturated fatty acids also antagonized thapsigargin-induced Ca^{2+} entry, suggesting that they may inhibit CCE [16]. In the present paper, we have studied the effects of AA and several AA analogs on CCE induced by emptying the intracellular Ca^{2+} stores with thapsigargin in rat thymocytes and in human neutrophils.

2. Materials and methods

Rat thymocytes [17] and human neutrophils [4] were obtained and handled as described previously. Cells were loaded with fura-2 by incubation with 4 μM fura-2/AM for 1 h at room temperature and $[Ca^{2+}]_i$ and was measured as described previously [4,17]. Briefly, cells were suspended at about 1% cytocrit and the fluorescence excited at 340, 360 and 380 nm (emission $>$ 510 nm) was measured simultaneously. $[Ca^{2+}]_i$ was estimated from the ratio of fluorescence excited at 340 and 380 nm [18]. Mn^{2+} was used as a tracer for Ca^{2+} entry [4,16]. The entry of Mn^{2+} was monitored by the quenching of fluorescence excited at 360 nm, which is insensitive to $[Ca^{2+}]_i$ variations [19]. The standard incubation medium had the following composition (in mM): NaCl, 150; KCl, 5; $MgCl_2$, 1; $CaCl_2$, 1; glucose, 10; sodium-HEPES, 10, pH 7.4. The experiments with thymocytes were performed at 25°C and those with neutrophils at 37°C.

For preparation of microsomes [20], rats (Wistar, 250 g) were killed by decapitation and the liver was homogenized in 5 vols. of a solution H containing 0.15 M KCl and 10 mM potassium-HEPES, pH 7.4 using a Potter homogenizer (400 rpm; 8 strokes). After a 15-min centrifugation at 17 000 × *g* and 4°C, the supernatant was recovered and centrifuged at 100 000 × *g* during 60 min at 4°C. The pellet was resuspended in 0.5 vols. of solution H (13 mg protein/ml) and stored at −80°C. For measurements of dealkylase activity, microsomes were diluted to 0.2 mg protein/ml in 0.1 M Tris/HCl buffer containing 50 μM NADPH and the assay was started by adding 0.5 μM 7-benzylresorufin. The activity was monitored by the increase of fluorescence (530 nm excitation, 585 nm emission) at room temperature [21].

Arachidonic acid was obtained from Menarini Diagnostici, Firenze, Italy. Staurosporin, phorbol 12,13-dibutyrate (PDB), miconazole, arachidic, 11-eicosenoic, 11,14-eicosadienoic and 8,11,14-eicosatrienoic acids (all *cis* forms) and arachidonic acid methyl ester (AME) were from Sigma, Madrid, Spain. 5,6-Dehydro-arachidonic acid (DHA), 5,8,11-eicosatriynoic acid (ETI) and 5,8,11,14-eicosatetraynoic acid (ETYA) were from Cascade Biochem Ltd., Reading, UK. Thapsigargin and arachidonyl trifluoromethylketone (AA-COCF3) were from Calbiochem, La Jolla, CA, USA. Other reagents were obtained either from Sigma or from E. Merck, Darmstadt, Germany.

3. Results

3.1. Arachidonic acid has a dual effect on Ca^{2+} entry

Fig. 1 shows the effects of arachidonic acid on $[Ca^{2+}]_i$ (upper panels) and on the entry of Mn^{2+} (lower panels), an index of Ca^{2+} entry [17,19], in rat thymocytes. The effects of two different concentrations, 5 and 50 μM, are shown. As described previously in several cell types [13], the higher concentration of AA increased $[Ca^{2+}]_i$ (Fig. 1A), and this was due to increased Ca^{2+} entry, as shown by the acceleration of Mn^{2+} entry (Fig. 1C). The smaller concentration of AA had apparently little or no effect (Fig. 1A,C). Stimulation of Ca^{2+} entry by AA was observed at concentrations above 20 μM and was maximal at 50–60 μM (results not shown). This concentration-dependence is similar to the one reported previously for platelets [13].

The right-hand panels in Fig. 1 illustrate the effects of AA in cells which had been previously

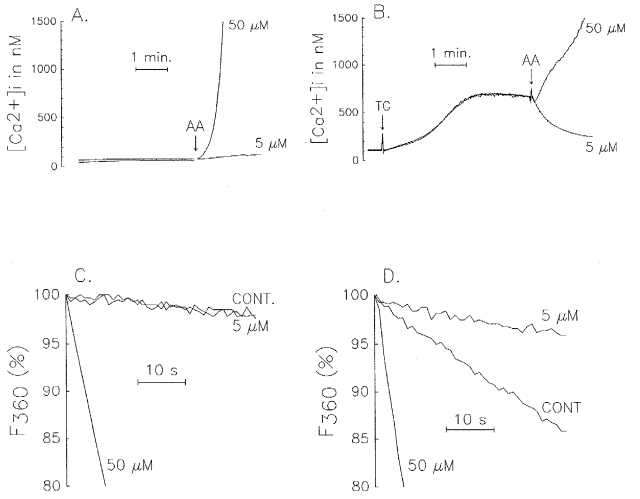

Fig. 1. Effects of arachidonic acid on $[Ca^{2+}]_i$ and on Mn^{2+} entry in rat thymocytes. The effects in control (A,C) and in thapsigargin-treated cells (200 nM, 5 min; B,D) are shown. Arachidonic acid was added to the cell suspension from a concentrated stock (14 mM) to give the concentrations shown. For Mn^{2+} entry measurements, 1 mM $MnCl_2$ was added from a concentrated (100 mM) stock and the decrease of the fluorescence excited at 360 nm (F_{360}) was followed. Readings have been normalized to 100% at the time of Mn^{2+} addition.

treated with the endomembrane ATPase inhibitor thapsigargin [6]. Thapsigargin treatment produced an immediate increase of $[Ca^{2+}]_i$ (Fig. 1B), which is due to Ca^{2+} leak from the intracellular Ca^{2+} stores to the cytosol. After a 2–3 min of treatment with thapsigargin, a plateau level with increased $[Ca^{2+}]_i$ is found at the steady-state. This reflects an increased Ca^{2+} entry, due to activation of CCE, which is balanced by increased Ca^{2+} extrusion at the plasma membrane, due to activation of plasma membrane Ca^{2+} ATPase by the increased $[Ca^{2+}]_i$ [8,9]. Inhibition of CCE at this stage produces a decrease of $[Ca^{2+}]_i$ towards levels near those found before treatment with thapsigargin [8]. The addition of 5 μM AA to thapsigargin-treated cells produced a decrease of $[Ca^{2+}]_i$, suggesting an inhibition of CCE (Fig. 1B).

The action of AA on CCE was further investigated by testing their effects on Mn^{2+} entry in thapsigargin-treated cells. We have previously shown that emptying the intracellular Ca^{2+} stores accelerates Mn^{2+} entry by 10–50 times in rat thymocytes [17]. Fig. 1D shows the effects of AA on thapsigargin-treated cells. The smaller concentration of AA (5 μM) inhibited the entry of Mn^{2+} by 73% (compare to 'Control', not treated with AA), confirming that AA is able to block CCE. In contrast, the higher (50 μM) concentration of AA produced a further increase of both $[Ca^{2+}]_i$ (Fig. 1B) and Mn^{2+} entry (Fig. 1D). Thus, AA has a dual effect on Ca^{2+} entry in rat thymocytes. At the higher concentrations, we confirm the previously reported stimulation of Ca^{2+} entry. At concentrations below 20 μM, it produced an inhibition of CCE. The concentration-dependence of this inhibition is illustrated in Fig. 2. Half-maximal inhibition was obtained between 1 and 3 μM AA for both, Mn^{2+} and Ca^{2+} entry. The inhibition of CCE by AA was prevented by adding bovine serum albumin (3 mg/ml) to the incubation medium (results not shown). The effect of arachidonic acid was greatly dependent on cell density, becoming much more active at the lower cell concentrations (results not shown).

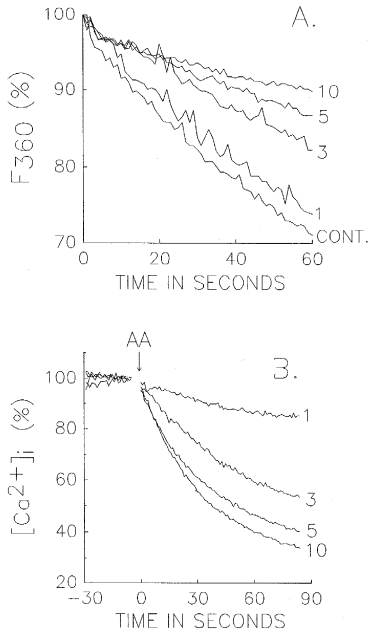

Fig. 2. Effects of different concentrations of arachidonic acid on Mn^{2+} uptake (A) and on $[Ca^{2+}]_i$ (B) in thapsigargin-treated thymocytes. Arachidonic acid concentrations, in μM, are indicated on the right. $[Ca^{2+}]_i$ is expressed as percent of the value at the time of arachidonic acid (AA) addition, 4 min after treatment with thapsigargin for standardization purposes. This value was 497 ± 19 nM (mean $\pm$ S.E.M.; $n = 13$) in this experiment. Other details as in Fig. 1.

3.2. Substrate specificity for the inhibition of CCE

The effects of arachidonic acid and several arachidonic acid analogs, all tested at 5 μM, on CCE of rat thymocytes are compared in Fig. 3. Both, the inhibition of the $[Ca^{2+}]_i$ increase induced by thapsigargin (open bars) and the inhibition of Mn^{2+} entry (cross-hatched bars) were measured. The saturated analog arachidic acid had no effect. The effect of the mono-unsaturated 11-eicosenoic acid was much weaker than AA, and the di- and tri-unsaturated arachidic acid derivatives had about the same effect as AA. Among the AA analogs containing triple bonds, 5,6-dehydro-arachidonic acid (DHA) and 5,8,11-eicosatriynoic (ETI) had about the same effects as AA and 5,8,11,14-eicosatetraynoic (ETYA) had consistently a somewhat smaller effect than AA. Arachidonic acid methyl ester (AME) had no effect. A non-metabolizable AA analog in which carboxylate group is replaced by the COCF3 group (AA-COCF3), widely used as phospholipase A_2 inhibitor [22], had no effect at 5 μM. When tested at 60 μM AA-COCF3 inhibited thapsigargin induced Mn^{2+} entry by 55 ± 8% (mean ± S.E.M.; $n = 5$).

In human neutrophils, arachidonic acid also antagonized CCE induced by thapsigargin, although concentrations about 3 times larger had to be used to achieve the same effects as in rat thymocytes. As with thymocytes, AA was more active at lower cell densities (results not shown). The effects AA several AA analogs on the increase of $[Ca^{2+}]_i$ and on Mn^{2+} entry are shown in Table 1. The relative actions were similar to those reported above for thymocytes, i.e., the saturated fatty acid had little effect, whereas the unsaturated ones inhibited CCE. Blocking the carboxylate group (AME) decreased the inhibitory effect.

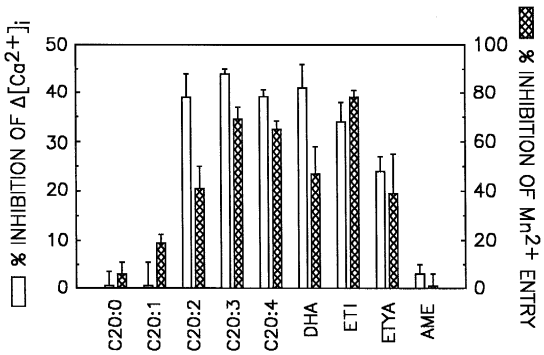

Fig. 3. Effects of several arachidonic acid analogs on the increase of $[Ca^{2+}]_i$ induced by thapsigargin (open bars) and on the entry of Mn^{2+} in thapsigargin-treated cells (cross-hatched bars). All the compounds were tested at 5 μM. Other details as in Fig. 1. C20:0, arachidic acid; C20:1, *cis*-11-eicosenoic acid; C20:2, *cis*-11,14-eicosadienoic acid; C20:3, *cis*-8,11,14-eicosatrienoic acid; C20:4, *cis*-5,8,11,14-eicosatetraenoic acid (arachidonic acid); DHA, 5,6-dehydroarachidonic acid; ETI, *cis*-5,8,11-eicosatriynoic acid; ETYA, *cis*-5,8,11,14-eicosatetraynoic acid; AME, arachidonic acid methyl ester. Each value is the average of 3–8 individual data. Vertical bars represent S.E.M.

Table 1
Inhibition of the increase of $[Ca^{2+}]_i$ induced by thapsigargin ($\Delta[Ca^{2+}]_i$) and of Mn^{2+} entry by arachidonic acid and other related substances in human neutrophils

Compound	Inhibition of $\Delta[Ca^{2+}]_i$	Inhibition of Mn^{2+} entry
Arachidic acid	8 ± 1 (3)	4 ± 2 (3)
11-Eicosenoic acid	4 ± 5 (3)	−1 ± 9 (3)
8,11,14-Eicosatrienoic acid	60 ± 1 (4)	53 ± 12 (4)
Arachidonic acid	53 ± 3 (9)	74 ± 7 (11)
Arachidonic acid methyl ester	13 ± 1 (3)	−2 ± 4 (4)

Concentrations were 15 μM. Values are given as percent inhibition (mean ± S.E.M.; the number of determinations is given in parentheses).

3.3. The effect of arachidonic acid is not reversed by staurosporin

Unsaturated fatty acids have been reported to activate protein kinase C [23,24]. Treatment with phorbol esters produces inhibition of CCE in human neutrophils, and this inhibition is relieved by the protein kinase C inhibitor staurosporin [8]. This result is confirmed in Fig. 4A–C. The right-hand panels in the figure illustrate the effects of staurosporin on the inhibition of CCE induced by AA. Staurosporin did not prevent the inhibition of the thapsigargin-induced $[Ca^{2+}]_i$ increase (Fig. 4D,E) nor the inhibition of the acceleration of the Mn^{2+} entry (Fig. 4F). In 8 similar experiments, staurosporin reverted the phorbol dibutyrate (PDB)-induced inhibition of the $[Ca^{2+}]_i$ increase from 68 ± 2 to 21 ± 2% (mean ± S.E.M.), whereas the inhibition by arachidonic was 53 ± 3 and 48 ± 3% with and without staurosporin, respectively. Regarding to the inhibition of Mn^{2+} entry the figures were (mean ± S.E.M.; $n = 6$; without and with staurosporin): PDB, 81 ± 2 and 20 ± 5%; arachidonic

acid 74 ± 7 and 73 ± 9%, respectively. In rat thymocytes, PDB had a very small effect on CCE. In any case, the inhibition of CCE of thymocytes by AA was not significantly modified by staurosporin (results not shown).

3.4. Unsaturated fatty acids inhibit P450-mediated dealkylase activity

ETYA has been reported to inhibit cytochrome P450-mediated activity [25] and a wide series of cytochrome P450 inhibitors were also blockers of CCE [17,26,27]. We have assayed the effects of AA and several related substances on the resorufin ether dealkylase activity of rat liver microsomes, a cytochrome P450-mediated activity [21]. Fig. 5A illustrates representative results. AA was strongly inhibitory, whereas arachidic acid had little effect. The effect of the P450 inhibitor miconazole is also shown for comparison. Table 2 compares the effects of arachidonic acid and some arachidonic acid analogs on the dealkylase activity. The parallel with the effects on CCE is apparent (compare to Fig. 3 and Table 1). Fig. 5B shows the correlation for the inhibitory actions on Ca^{2+} influx through CCE in rat thymocytes and on microsomal dealkylase activity. The correlation coefficient, *r*, was 0.87. Similarly, the correlation coefficient for inhibition of Mn^{2+} entry through CCE and dealkylase activity was 0.76 (results not shown).

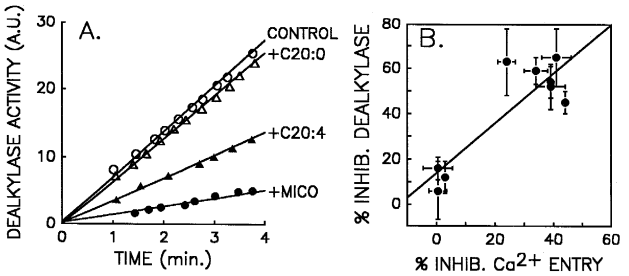

Fig. 5. Effects of fatty acids on benzylresorufin-dealkylase activity of rat liver microsomes. A: comparison of the effects of arachidic (C20:0) and arachidonic acid (C20:4), both at 15 μM, on dealkylase activity. The effect of miconazole (10 μM) is shown for comparison. B: correlation between the effects of several fatty acids (same as in Fig. 3 and Table 2) on dealkylase activity and on the increase of $[Ca^{2+}]_i$ induced by thapsigargin in rat thymocytes. Bars represent S.E.M. Line adjusted by the minimum squares procedure.

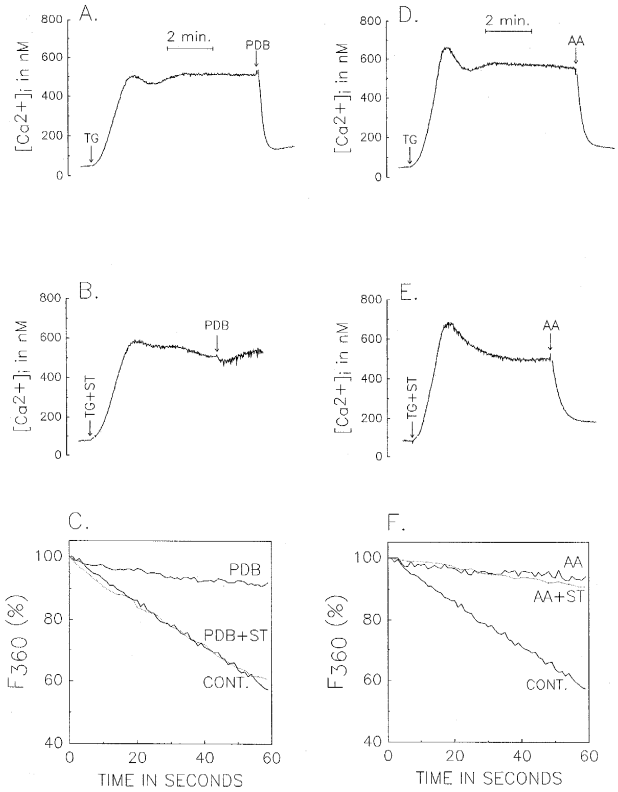

Fig. 4. Comparison of the effects of staurosporin (ST) on the inhibition of capacitative Ca^{2+} entry induced by either phorbol 12,13-dibutyrate (PDB) or by arachidonic acid (AA) in human neutrophils. The concentrations of thapsigargin, PDB and AA and staurosporin were 200 nM, 100 nM, 15 μM and 200 nM, respectively. The concentration of Mn^{2+} was 0.2 mM. Other details as in Fig. 1.

Table 2
Effects of several fatty acids on 7-benzylresorufin-dealkylase activity of rat liver microsomes

Compound	% Inhibition
Arachidic acid	13 ± 17 (4)
11-Eicosenoic acid	16 ± 5 (5)
11,14-Eicosadienoic acid	52 ± 10 (3)
8,11,14-Eicosatrienoic acid	45 ± 5 (4)
Arachidonic acid (5,8,11,14)	54 ± 7 (5)
5,6-Dehydroarachidonic acid (DHA)	65 ± 15 (2)
5,8,11-Eicosatriynoic (ETI)	59 ± 6 (3)
5,8,11,14-Eicosatetraynoic (ETYA)	50 ± 10 (3)
Arachidonic acid methyl ester	17 ± 7 (5)

All the compounds were tested at 15 μM, except DHA, which was tested at 5 μM. Values are given as mean ± S.E.M. The number of determinations is given in parentheses.

4. Discussion

The results shown here demonstrate that arachidonic acid, in addition to its previously described Ca^{2+}-influx-stimulating effect [13], is able, at lower concentrations, to inhibit capacitative Ca^{2+} entry in rat thymocytes and in human neutrophils. In several cell lines, the receptor-operated Ca^{2+} influx is secondary to activation of CCE on emptying of the intracellular Ca^{2+} stores [4]. Therefore, our results are consistent with and may help to explain previous reports on inhibition of receptor-operated Ca^{2+} influx by unsaturated fatty acids [15,16]. It has been claimed that the inhibition of Ca^{2+} influx by fatty acids may be due to increased Ca^{2+} extrusion [14,28,29]. The experiments with Mn^{2+} shown here demonstrate, however, a direct inhibitory effect on Ca^{2+} entry. The inhibitory effect on CCE is not specific for arachidonic acid, but it is shared by other unsaturated analogs (specially those containing more than two double or triple bonds), but not by the saturated ones. The free carboxylate group is required. Surprisingly, the substrate specificity profile for the inhibitory effect is similar to the one reported before for the Ca^{2+}-influx stimulating effect of several fatty acids [13], although the stimulatory effect requires a concentration about 10 times larger ([13], see also Fig. 1). Since large amounts of arachidonic acid may be produced on stimulation of phospholipase A_2 by a wide variety of agonists, regulation of Ca^{2+} influx by fatty acids could have physiological and pathophysiological relevance.

It has been recently proposed that production of 5,6-epoxy-eicosatrienoic acid (EET), whose synthesis from AA is catalyzed by a microsomal P450 monooxygenases, is stimulated by emptying the Ca^{2+} stores and that EET stimulates CCE. Thus, EET might act as the messenger for activation of CCE [12]. Our results do not favor, in principle, this possibility since, under this view, AA should not be expected to inhibit, but to stimulate, CCE. It could be argued that fatty acids or their derivatives may antagonize the synthesis of EET and this possibility cannot be excluded. In any case, our results suggest that the inhibitory effect is due to the fatty acid itself and not to a metabolite derived from it, as we find that several non-metabolizable AA analogs also inhibit CCE. As a matter of fact, some of these analogs are inhibitors of arachidonic acid metabolism. On the other hand, we have shown before that neither cyclooxygenase (acetyl-salicylic acid, compound BW755C) nor lypooxygenase inhibitors (compound BW755C, baicalein, caffeic acid, esculetin, phenidone) prevent CCE in rat thymocytes [17,26]. 14,15-Epoxyeicosatrienoic acid has been recently shown to produce inhibition of CCE in human platelets [30].

It has also been recently shown that activation of PKC using phorbol esters produces inhibition of CCE in human neutrophils [8]. Since unsaturated fatty acids have been reported to activate PKC [23,24] it could be proposed that the inhibition of CCE shown here takes place by this mechanism. We find, however, that whereas the PKC inhibitor staurosporin was able to prevent the inhibition of CCE produced by phorbol ester, it had little or no effect on the inhibition produced by AA. This suggests that inhibition of CCE by fatty acids do not take place via activation of PKC. We have recently shown that the inhibition of CCE of human neutrophils produced by the chemotactic peptide fMLP [8], which involves protein phosphorylation [7], is not reverted by staurosporin either [8]. In addition, the appearance of inhibition of CCE by phorbol ester and by fMLP during differentiation of HL60 human leukemia cells towards granulocytes shows a different time course [9]. This suggests that protein kinases other than the C-type may down-regulate CCE. Thus, the possibility that unsaturated fatty acids may act through other protein kinases may not be excluded.

On the basis of the inhibitory effects of a large series of cytochrome P450 antagonists, we have proposed that the link between emptying of the intracellular Ca^{2+} stores and activation of CCE may be a metabolite generated by a microsomal cytochrome P450 whose activity would be stimulated by the decrease of $[Ca^{2+}]_i$ inside the stores [17,26]. This hypothesis has been severely criticized on the basis of what we consider as circumstantial evidence [31–33]. ETYA, one of the arachidonic acid analogs tested here, had been previously reported to inhibit P450-mediated activity [25]. We now find that the same fatty acids that antagonize CCE also inhibit rat liver microsomal benzylresorufin-dealkylase, a P450-mediated activity. This might be casual, but certainly it is consistent with the P450 hypothesis. We find other similarities among the unsaturated

fatty acids tested here and other P450 inhibitors shown before to inhibit CCE. For example, many of them are lypooxygenase inhibitors (ETYA, ETI or DHA among arachidonic acid derivatives, nordihydroguaiaretic, gossypol or compound AA861 among P450 inhibitors [17,26]). On the other hand, the ability to increase Ca^{2+} (and Mn^{2+}) influx found for the fatty acids at the higher concentrations (Fig. 1 and [13]) has also been reported for several P450 inhibitors when used at 5–10 times the IC_{50} for inhibition of CCE (econazole, miconazole, nordhydroguaiaretic acid, compound SKF525A, gossypol [27]). In all the cases the Ca^{2+} entry is blocked by Ni^{2+}. Of course, these results are only phenomenological evidence in favor of the P450 hypothesis for the regulation of CCE. On the other hand, the P450 family is so wide and with such different functions [34,35] that, without further concretion, it is difficult to valorate adequately the arguments for and against this hypothesis. Therefore, it seems reasonable to wait for direct evidence on the mechanism linking the intracellular Ca^{2+} stores and CCE before giving a definitive interpretation to the inhibition by P450 inhibitors and by unsaturated fatty acids.

Acknowledgements

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