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Biochemical Pharmacology 67 (2004) 903–909
Biochemical Pharmacology
www.elsevier.com/locate/biochempharm

Ca^{2+} influx is not involved in acute cytotoxicity of arachidonic acid

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Received 27 August 2003; accepted 13 October 2003

Abstract

Arachidonic acid (AA; 20:4, n-6) has been implicated in cell damage in the brain under ischemia–reperfusion and other pathological conditions. In our experiments, PC12 cells exposed to >10 μM AA died within 1–2 hr, as assessed by the LDH release assay. Since AA is known to induce Ca^{2+}/cation-permeable conductance in the plasma membrane, we investigated whether Ca^{2+} influx plays a role in this acute cell death. We found that extracellular Ca^{2+} was not required for the toxic effect of AA. In fact, the removal of extracellular Ca^{2+} dramatically accelerated its development: the half-time of the toxic effect of 40 μM AA decreased from 70.1 ± 0.3 min in the presence of 5 mM Ca^{2+} to 7.4 ± 0.3 min in the Ca-free solution. The extent of cell killing depended only weakly on AA concentration and ion composition, remaining within the 70–95% range. The AA-induced acute death was not affected by inhibitors of AA metabolism (nordihydroguaiaretic acid, indomethacin, proadifen), whereas some antioxidants tested (deferoxamine and ellagic acid), but not all (melatonin), partially suppressed it. Also, it was not affected by changes in the extracellular ionic strength or mimicked by an acetylenic analog of AA 5,8,11,14-eicosatetraynoic acid. We conclude that lethal injuries sustained by cells during short exposures to AA were caused by the fatty acid itself and were not mediated by the AA-induced influx of Ca^{2+}/cations. Moreover, direct physical effects of AA on the plasma membrane (changes in membrane fluidity or detergent-like action) were also excluded.

Keywords: Cell death; Fatty acids; Calcium; Noncapacitative Ca entry; Nonselective cation channels

1. Introduction

AA (20:4, n-6) is an essential polyunsaturated fatty acid that plays an important role in intracellular and intercellular signal transduction in the nervous system, both as a source of biologically active metabolites—prostaglandins, leukotrienes, and thromboxanes, and also by exerting direct biological effects (reviewed in [1,2]).

Accumulated evidence shows that free AA, liberated from membrane phospholipids by the activities of phospholipases A_2 and C, as well as AA metabolites also play a significant role in cell damage in the brain under ischemia and other pathological conditions (reviewed in [2–5]). The role for AA in pathology of the brain was first suggested by Bazan and colleagues, who demonstrated that massive amounts of AA and other fatty acids are released during brain ischemia and convulsion (reviewed in [6]). In another exemplary study, the levels of free AA in the impact-injured spinal cord have been found to exceed 20 times the control values 6 hr after the initial injury [7].

The mechanisms of AA toxicity appear to be complex and are not fully understood. Oxidative metabolism of AA generates not only biologically active eicosanoids but also reactive oxygen species, which augment cell damage by attacking proteins and promoting lipid peroxidation [2,5]. Oxygen radicals have been identified as the primary toxic agents in the death of cultured hippocampal [8] and spinal cord neurons [9] caused by prolonged action of low levels of exogenous AA, but were ruled out as the cause of a more rapid form of cell death caused by higher AA concentrations in neuronally differentiated NB-104 cells [10]. Significant attention received also the deleterious effects of AA on mitochondria (e.g. [11]), which emerged as key players in the regulation of cell death [12]. Recent studies have identified the induction of mitochondrial permeability transition as the cause of AA-induced death in rat hepatoma MH1C1 cells [13,14].

Another pathway with a significant potential to contribute to AA toxicity is through the intracellular Ca^{2+} regulation [10]. An overload of the cellular systems

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Abbreviations: AA, arachidonic acid; 5,8,11,14-ETYA, eicosatetraynoic acid; *cis*-4,7,10,13,16,19-DHA, docosahexaenoic acid; NMDG, *N*-methyl-D-glucamine; DMSO, dimethyl sulfoxide.

0006-2952/$ – see front matter © 2003 Elsevier Inc. All rights reserved.
doi:10.1016/j.bcp.2003.10.005

designed to control the intracellular Ca^{2+} levels within narrow limits is a convergence point for many modes of cell death [15,16]. Arachidonic acid can trigger the release of Ca^{2+} from intracellular stores [17–20] and is also known to affect the activity of a variety of ion channels in the plasma membrane (reviewed in [21]). Recently, AA was shown to induce noncapacitative Ca^{2+} entry in many cell types [17,19,22,23]. The latter effect may be a more general phenomenon, whereby arachidonic acid activates Ca^{2+}/cation-permeable channels belonging to the transient receptor potential-like (TRP) family (e.g. [24]). An influx of extracellular Ca^{2+} ions via the AA-induced conductance (e.g. [17,19]) may aggravate the mechanisms of cell injury and, in particular, accelerate the time of onset of mitochondrial permeability transition [25,26].

We were interested in investigating the role of the AA-induced transmembrane Ca^{2+}/cation influx in cell death that occurs during short exposures to micromolar concentrations of the fatty acid. The results presented in this report suggest that the toxic effect of >10 μM AA was caused by arachidonate itself, without involvement of its metabolites, and was not mediated by the AA-induced influx of the extracellular Ca^{2+}/cations.

2. Materials and methods

2.1. Cell culture

The experiments were carried out on cultured PC12 cells that are often used as model nerve cells. The cells were grown in RPMI-1640 medium supplemented with 10% fetal bovine serum, 5% heat-inactivated horse serum and antibiotics, in a humidified 5% CO_2/air mixture in a CO_2 incubator. For toxicity studies, PC12 cells were seeded in 24-well plates at $\sim 2 \times 10^4$ cells/well and grown for 1–2 days.

2.2. Treatment and cytotoxicity determination

The arachidonate-induced cell death was assessed quantitatively by measuring the activity of lactate dehydrogenase (LDH) released from damaged cells into the extracellular fluid using the LDH assay kit (Roche Molecular Biochemicals). Since all treatments lasted less than 4 hr, no significant changes in the number of cells in control and test wells were expected that would have complicated the use of the LDH cell death assay. All wells on a plate were washed twice with a control solution (the one to which test substances were added in a particular experiment). Then, triplicate well groups were filled, one each with the control solution and 0.2% Triton X-100 in the control solution, and the rest six groups with various test solutions (the control solution containing AA and/or other tested agents). After cells have been exposed to the above solutions in a CO_2 incubator at 37°, the extracellular medium was collected separately from each well and centrifuged for 10 min at 250 *g* to remove cell debris. Aliquots (100 μL) of the medium were then combined with equal volumes of the LDH assay mixture in a 96-well assay plate and incubated for 30 min at room temperature. The reaction was stopped by addition of 1 M HCl. LDH activity was assessed as a difference in absorbance at 492 and 690 nm using a microplate reader (MRX, Dynatech Laboratories). The extent of cell death in the test wells (six triplicate groups) was calculated as percentages of the difference in absorbance between the Triton wells (100% cell death) and the control wells (0% cell death). Since the LDH assay is performed on samples containing test substances, the lack of their direct effects on LDH activity was verified routinely by inclusion of these substances in control wells and in some cases also in wells with Triton.

2.3. Solutions and chemicals

The basic extracellular solution contained (mM): $CaCl_2$ 1.26, $MgSO_4$ 0.8, KCl 5.36, KH_2PO_4 0.44, $NaHCO_3$ 4.17, NaCl 137, Na_2HPO_4 0.34, glucose 12.5, HEPES 10, pH 7.4 with NaOH. In solutions with 5 mM $CaCl_2$ (5-Ca solution) or 10 mM $CaCl_2$ (10-Ca solution) the concentration of NaCl was reduced accordingly. In the Ca-free solution, $CaCl_2$ was replaced with NMDG. The cation-free solution contained (mM): NMDG 140, glucose 12.5, HEPES 10, pH 7.4 adjusted with HCl (also referred to as the high ionic strength solution). In the low ionic strength solution, the NMDG concentration was lowered to 50 mM and the osmolarity was adjusted with sucrose. The osmolarity of all solutions was kept at 300–310 mOsm. Stock solutions of arachidonic acid (5,8,11,14-eicosatetraenoic acid, sodium salt), 20 mM in water, DHA and ETYA, both at 100 mM in DMSO, were kept aliquoted at −20° until use. Each aliquot was used only for one experiment, and the remaining fatty acid was discarded to minimize autoxidation. Proadifen hydrochloride (SKF-525A, α-phenyl-α-propylbenzeneacetic acid 2-(diethylamino)ethyl ester) and deferoxamine mesylate salt were dissolved in water. Ellagic acid (4,4′,5,5′,6,6′-hexahydroxydiphenic acid dilactone), indomethacin, and nordihydroguaiaretic acid (NDGA), were all dissolved in DMSO. Final concentrations of DMSO in the test solutions did not exceed 0.1%. Melatonin (*N*-acetyl-5-methoxytryptamine) was dissolved in ethanol. All chemicals were from Sigma.

2.4. Statistics

Values are means ± SEM of the indicated numbers (at least three) of independent experiments performed in triplicate. The significance of differences between experimental data groups was determined using Student's *t*-tests (GraphPad Prism 3.03, GraphPad Software). A $P < 0.05$ was considered significant. The *P* values are indicated in

the figures as follows: $P < 0.05$ (*), $P < 0.001$ (**), $P < 0.0001$ (***).

3. Results

3.1. Toxicity of arachidonic acid in PC12 cells and the role of extracellular cations

In earlier experiments, we have tested how short, 1 hr-long exposures to different concentrations of arachidonic acid from 0.1 to 20 μM affected viability of PC12 cells in the 24 hr period following exposure. We found that whereas no deleterious effect on neuronal survival was observed with AA concentrations below 10 μM, a significant number of cells exposed to 20 μM AA were killed (data not shown). With higher concentrations of arachidonate, the toxic effect developed so rapidly, that many exposed cells actually died during the 1 hr exposure periods, e.g. approximately 75% at 100 μM AA (shown by black bars in Fig. 1). Delayed cell death, assessed 24 hr later (white bars in Fig. 1), accounted for a progressively smaller part of total cell death as AA concentration increased, largely due to increasing contributions of early cell death. In the experiments described below we have investigated peculiarities of this rapidly progressing, acute phase of AA-induced cell death (acute cytotoxicity).

Since one of the main objectives of the present study was evaluating the role of the AA-activated Ca^{2+}/cation-permeable conductance [17,19,22,23], we investigated how ion composition of the extracellular medium affected acute cytotoxicity of arachidonic acid. If the Ca^{2+}/cation influx is involved, its modulation by increasing or decreasing extracellular Ca^{2+} would likely result in enhancement or suppression of AA-induced cell death. We found that, contrary to these predictions, the complete withdrawal of extracellular Ca^{2+}, or of all extracellular cations including

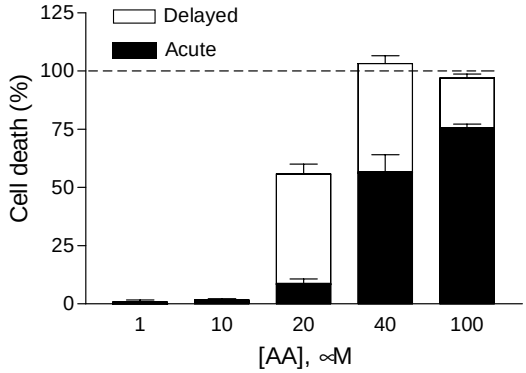

Fig. 1. Acute and delayed phases of arachidonic acid-induced cell death. The percentages of PC12 cells killed during (black bars) and 24 hr after (white bars) a 1 hr exposure to different AA concentrations in the 1.26-Ca solution (determined as the amount of LDH released into the bath at these time points in relation to the LDH released into Triton-treated wells).

Ca^{2+}, did not suppress AA toxicity. Furthermore, under these conditions the toxic effects of arachidonate developed faster. Figure 2 shows the kinetics of cell death caused by exposure to 20 μM AA (Fig. 2A) and 40 μM AA (Fig. 2B) in solutions with different ion composition. For the purpose of comparison, we used the duration of exposure required for the 50% extent of cell death observed under particular conditions. This parameter (T_{50}, see Fig. 2A) was determined by approximating experimental data with Boltzmann sigmoid curves. Whereas exposure to higher concentrations of AA killed PC12 cells faster in all solutions used, the largest effect on the half-time of the acute toxic effect of arachidonate was that of Ca^{2+} ions. When compared with the 5-Ca solution, withdrawal of extracellular Ca^{2+} resulted in an almost 2-fold decrease in T_{50}, from 74 to 41 min, for 20 μM AA and in an 10-fold decrease in T_{50}, from 70 to 7 min, for 40 μM AA. Removal of monovalent cations or the 2-fold increase in Ca^{2+}

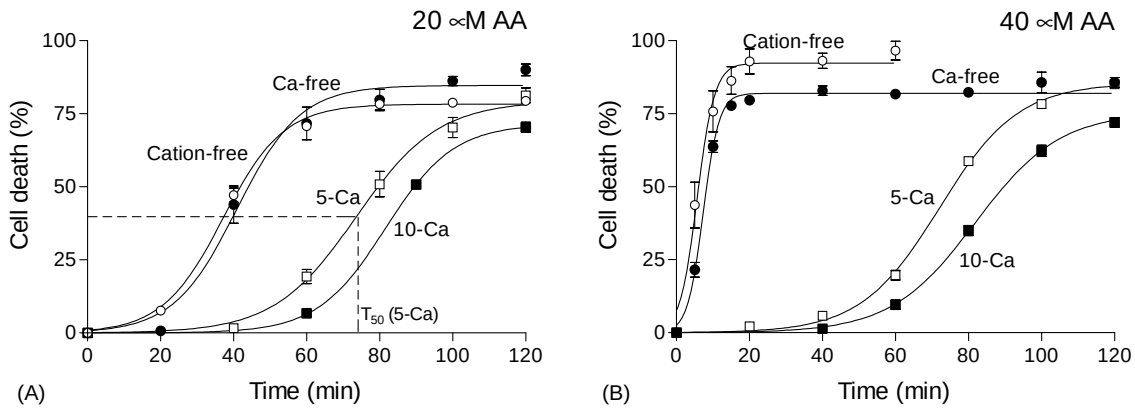

Fig. 2. The effects of extracellular Ca^{2+} on the time course of acute toxic effects of AA. (A) Temporal development of cell death caused by exposure of PC12 cells to 20 μM AA in the cation-free (○), Ca-free (●), 5-Ca (□), and 10-Ca (■) solutions. The continuous lines were obtained by fitting the experimental results to the Boltzmann equation (GraphPad Prism 3.03). The T_{50} values are: 37.1 ± 0.8, 40.9 ± 2.3, 73.6 ± 1.5, and 81.6 ± 1.6 min, respectively. (B) Same for 40 μM AA. The T_{50} values determined for the four solutions (in the same order) are as follows: 5.7 ± 0.6, 7.4 ± 0.3, 70.1 ± 0.3, and 81.9 ± 0.8 min. Each data point is mean ± SEM, with N = 3–5. Standard errors for T_{50} values are given as determined by the fitting procedure.

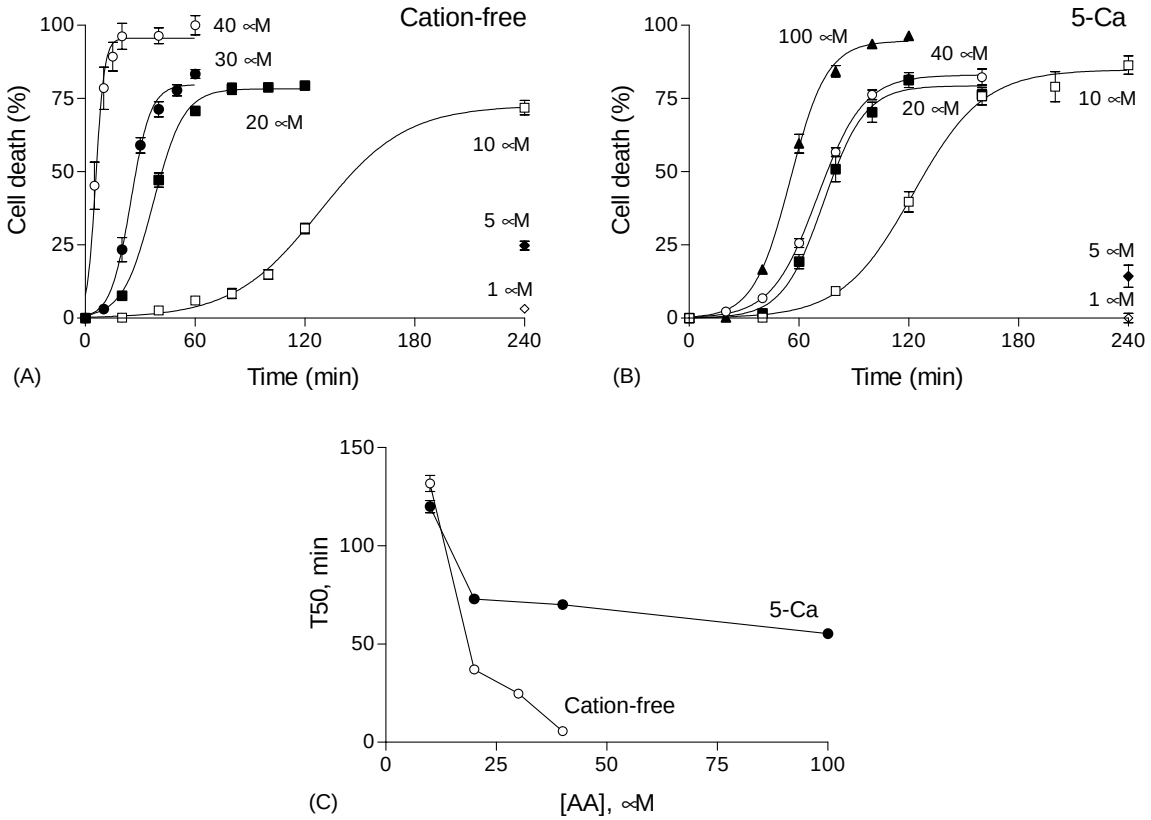

Fig. 3. The concentration-dependence of the kinetics of AA toxicity. (A) Temporal development of cell death caused by exposure of PC12 cells to 10 (□), 20 (■), 30 (●), and 40 μM AA (○) in the cation-free solution. The continuous lines were obtained as described in the legend to Fig. 2. The respective T_{50} values were used to plot graph in (C). To allow for a more complete development of the toxic effect, the duration of cell exposure to 10 μM AA was increased to 4 hr. The percentages of cells killed during 4 hr-long exposures to 1 and 5 μM AA are also shown. (B) Same for the 5-Ca solution. The AA concentrations used were: 10, 20, 40, and 100 μM. Each data point is mean ± SEM, with N = 3–5. (C) The relationships between T_{50} values and AA concentration for the cation-free (○) and the 5-Ca (●) solutions were constructed from the data shown in graphs A and B, respectively. Standard errors for T_{50} values are shown as determined by the fitting procedure.

concentration, from 5 to 10 mM, caused much smaller changes in the T_{50} values.

These findings were corroborated by the results of another study, in which the temporal parameters of the toxic effects of different AA concentrations (from 10 to 100 μM) were compared in the cation-free (Fig. 3A) and the 5-Ca solutions (Fig. 3B). These two solutions provided respectively for the fastest and the slowest kinetics of the AA-induced cell death. In the cation-free solution, the T_{50} values observed with 10 and 40 μM AA differed more than 20-fold, decreasing from 132 to 6 min, respectively (Fig. 3C). In contrast, in the 5-Ca solution, the difference in the T_{50} for 10 and 100 μM AA was much smaller, only a 2-fold decrease from 120 to 55 min (Fig. 3C). Interestingly, the time course of the toxic effects of 10 μM AA was similar in both solutions; the largest differences in the T_{50} values for the two solutions were observed with higher concentrations of arachidonate (see also Fig. 2).

In contrast to the large changes in the kinetics of AA toxicity due to changes in AA concentration or ion composition of the extracellular solutions, the maximal extent of cell death caused by 20–100 μM AA during 2 hr-long exposures or by 10 μM AA during 4 hr-long exposure varied much less, remaining within the 70–95% range. On the other hand, the toxicity of 1 and 5 μM AA, even with 4 hr-long exposures, was significantly lower in both solutions.

3.2. The role of AA metabolism and of oxidative stress

Many of the reported effects of arachidonic acid have been attributed to various products of its metabolism (eicosanoids) by three major oxygenases—lipoxygenases, cyclooxygenases, and epoxygenases (e.g. [2]). To test for the role of AA metabolism in the toxic effects, we studied how inhibitors of the oxygenases affected AA-induced cell death. The inhibitors used were ETYA (20–40 μM), a nonmetabolyzable AA analog which inhibits all three major pathways of AA metabolism by acting as a false substrate [27], as well as nordihydroguaiaretic acid, indomethacin, and proadifen, the specific inhibitors of 5-lipoxygenase, cyclooxygenase and cytochrome P450, respectively [27–29]. Each of the inhibitors was tested at 1, 2, 5, and 10 μM concentrations. None of these substances protected PC12 cells from AA-induced death (Fig. 4A), which argues against a substantial contribution of eicosanoids to acute cytotoxicity of arachidonate.

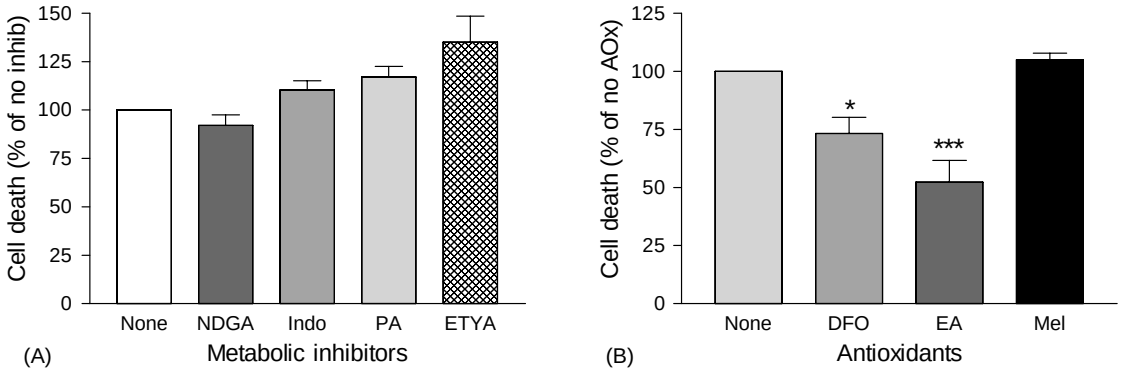

Fig. 4. The effects of metabolic inhibitors and of antioxidants. (A) The percentages of PC12 cells killed during 2 hr exposures to 40 μM AA in the absence of inhibitors (None)—designated as 100%, and in the presence of 10 μM NDGA (NDGA), 10 μM indomethacin (Indo), 10 μM proadifen (PA), or 20 μM ETYA (ETYA). (B) The percentages of cells killed during 2 hr exposures to 40 μM AA in the 1.26-Ca solution in the absence of antioxidants (None)—designated as 100%, and in the presence of 100 μM deferoxamine (DFO), 250 μM ellagic acid (EA), or 250 μM melatonin (Mel). The cells were pre-incubated in the presence of each of the antioxidants for 1 hr before the arachidonate was added. Data presented as a mean ± SEM (N = 3).

The metabolic conversions of arachidonic acid are accompanied also by generation of toxic reactive oxygen species (e.g. [2]). The role of oxygen radicals was examined by using several reputed antioxidants, namely, deferoxamine [30], ellagic acid [31], and melatonin [32]. To ensure that the antioxidants reached the intracellular space, cells were pre-incubated for 1 hr in the presence of each of the substances (100 μM deferoxamine, 250 μM ellagic acid, 250 μM melatonin) before being exposed for additional 2 hr to 25 μM arachidonic acid, also in the presence of the antioxidants (in the concentrations indicated). As shown in Fig. 4B, deferoxamine and ellagic acid had significant cytoprotective effects, whereas melatonin did not protect PC12 cells from AA-induced death.

3.3. Direct physical effects on the plasma membrane are not responsible for AA toxicity

It is known that polyunsaturated fatty acids, including arachidonic acid, are able to perturb the order of the membrane lipids, thereby inducing changes in membrane fluidity. We tested ETYA and DHA, two polyunsaturated fatty acids for which experimental data are available concerning their effects on membrane fluidity, for their effects on viability of PC12 cells. Although AA and ETYA have been shown to cause similar changes in membrane fluidity [36,37], exposure of PC12 cells for 1 hr to 20–100 μM ETYA in the absence of arachidonic acid did not cause cell death (−2 ± 1% killed cells at 100 μM ETYA). The cytotoxic effect of DHA resembled that of arachidonic acid, albeit at higher concentrations; the extent of cell death caused by 1 hr-long exposure to 100 μM DHA in the cation-free solution was 94 ± 2% (N = 3) of that observed with 40 μM AA under similar conditions. At 40 μM, DHA was only marginally toxic to PC12 cells.

Another physical action of fatty acids on the plasma membrane is referred to as a detergent-like effect and is associated with formation of micelles. Micelles are aggregates of fatty acid molecules, formed because of their poor solubility in aqueous solutions, and are believed to be able to disrupt cell membranes and/or create lipidic pores permeable to Na^+ and Ca^{2+} ions (e.g. [33]). Formation of micelles is enhanced by the presence of cations, especially Ca^{2+}, and by higher ionic strength [34]. The effects of extracellular Ca^{2+} on cytotoxicity of AA described in the preceding sections are not consistent with the anticipated role of micelles in mediating AA-induced cell death. Also, it was not supported by the results of experiments with varying the ionic strength of AA-containing solutions; there was no significant difference in the extent of cell death caused by exposure of PC12 cells to 40 μM AA for 2 hr or to 20 μM AA for varying periods of time (0.5, 1, and 1.5 hr) between solutions with the low or the high ionic strength (data not shown).

4. Discussion

The data presented here indicate that exposure to micromolar levels of polyunsaturated fatty acids, such as arachidonic and docosahexaenoic acids, even for short periods of time can have deleterious consequences for cell viability. In our experiments, 1–2 hr-long exposures of PC12 cells to >10 μM AA resulted in extensive cell death evident immediately after the treatment. A similar acute toxic effect of arachidonic acid has been described in differentiated NB-104 cells and suggested to be caused by AA-induced cytoplasmic Ca^{2+} overload [10]. The excessive Ca^{2+} could be released from the intracellular stores [10], but also could enter from the extracellular space. The acute phase of AA toxicity occurs within the concentration range, 10–100 μM, at which arachidonate is known to affect ion channels (reviewed in [21]) and, in particular, to induce an ion conductance in the plasma membrane that is permeable to Ca^{2+} [17,19,23] or to monovalent cations in the absence of Ca^{2+} [22]. The results of the present

study, notably the failure to suppress AA-induced cell death by removal of extracellular Ca^{2+} or Ca^{2+} together with monovalent cations, argue against a significant role of the putative influx of Ca^{2+}/cations in acute AA toxicity. It remains unclear at present whether it is because the AA-induced Ca^{2+} release alone is sufficient to initiate cell death or whether there is a Ca-independent pathway whereby arachidonic acid, applied in higher concentrations, affects cell viability.

Changes in extracellular Ca^{2+}, however, had a significant impact on the speed of development of AA-induced cell death; whereas the removal of extracellular Ca^{2+} led to its acceleration, an increase in the Ca^{2+} concentration delayed it. Furthermore, in the presence of high Ca^{2+} concentrations, the relationship between the kinetics of AA toxicity and the concentration of arachidonate was greatly weakened, if compared with that seen in the Ca-free or the cation-free solutions. If judged by the observations in the cation-free solution, the T_{50} value appears to be a sensitive indicator of the cytotoxic AA concentration. Extending this relationship to the 5-Ca solution, the cytotoxic AA concentration in this solution with 100 μM AA must have been less than the cytotoxic AA concentration in the cation-free solution with 20 μM AA. Such a situation could arise because of a limited solubility of arachidonate in Ca^{2+}-containing solutions (see [1]). It would appear that at low concentrations, up to 10 μM, most of arachidonic acid added to the 5-Ca solution was unaffected by the presence of Ca^{2+}, as indicated by the proximity of the T_{50} values for 10 μM AA in this and in the cation-free solutions (Fig. 3C), whereas addition of higher AA concentrations, up to 100 μM, resulted in only a moderate increase in the cytotoxic AA concentration due to formation of insoluble calcium salts of arachidonate.

The acute phase of AA toxicity is likely to be caused by arachidonate itself rather than by its metabolites, since the inhibitors of all three major pathways of AA metabolism were ineffective in preventing the acute cytotoxicity. These findings (see also [10]) agree with the reported observation that metabolic conversion of exogenous AA added to neuronal cultures was negligible during the first 6 hr [9]. The significance of the time factor is emphasized also by the prominent role of AA metabolism and accompanying generation of the reactive oxygen species in longer-term experiments (48–72 hr), in which exposure to <10 μM AA resulted in cell death [8,9].

Our findings concerning the role of the reactive oxygen species do not allow us to reach an unequivocal conclusion. Two of the antioxidants used, ellagic acid and deferoxamine, partially protected PC12 cells from AA-induced death, whereas melatonin did not. Obviously, the particular mechanisms of action of each of the antioxidants determine their effects on the cell death pathways [8–10].

Lipophilic substances such as fatty acids may affect cells through direct actions on plasma membrane (e.g. [35]). The two mechanisms most often considered are changes in membrane fluidity and the detergent-like effect. Our observations contradict the role of these physical effects in acute AA toxicity. The notable lack of toxicity of ETYA (see also [36]), which has been shown to increase fluidity of cell membranes similarly to arachidonic acid [36,37], argues strongly against the involvement of this mechanism. The detergent-like effect of fatty acids on plasma membrane is believed to be due to formation of micelles, globular aggregates of fatty acid molecules, which disrupt the lipid bilayer upon insertion. Formation of micelles becomes prominent at arachidonate concentrations exceeding a certain threshold, the so-called critical micelle concentration [34]. It is known that the aggregate state of fatty acids is particularly sensitive to the presence of Ca^{2+} and is also enhanced by higher ionic strength [34], which may explain widely divergent reported values for the critical micelle concentration for arachidonic acid, ranging from ∼10 to 150 μM (e.g. [21]). The enhancement of AA toxicity in the absence of Ca^{2+}, as well as the lack of a significant effect of varying the ionic strength of AA-containing solutions on AA-induced cell death, observed in our study, do not support the perceived role of micelle formation as the cause of cell injuries.

In conclusion, the data presented suggest that acute death of PC12 cells during short exposures to micromolar levels of arachidonic acid was caused by the fatty acid itself and did not involve an influx of extracellular Ca^{2+}/cations. The observed effects of extracellular Ca^{2+} on the kinetics of AA-induced cell death may be explained by formation of insoluble calcium salts of arachidonate, with the resultant decrease in AA toxicity. We conclude also that nonspecific perturbations of the plasma membrane did not play a noticeable role in acute toxicity of arachidonic acid.

Acknowledgments

This work was supported by grants from Ontario Neurotrauma Foundation and Canadian Institutes of Health Research.

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