Original article

Regulation of intracellular calcium levels by polyunsaturated fatty acids, arachidonic acid and docosahexaenoic acid, in astrocytes: possible involvement of phospholipase A_2

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Abstract – Pathological conditions in the brain, such as ischemia, trauma and seizure are accompanied by increased levels of free n-6 and n-3 polyunsaturated fatty acids (PUFA), mainly arachidonic acid (AA, 20:4n-6) and docosahexaenoic acid (DHA, 22:6n-3). A neuroprotective role has been suggested for PUFA. For investigation of the potential molecular mechanisms involved in neuroprotection by PUFA, we studied the regulation of the concentration of intracellular Ca^{2+} ($[Ca^{2+}]_i$) in rat brain astrocytes. We evaluated the presence of extracellular PUFA and the release of intracellular PUFA. Interestingly, only the constitutive brain PUFA AA and DHA, but not eicosapentaenoic acid (EPA) had prominent effects on intracellular Ca^{2+}. AA and DHA suppressed $[Ca^{2+}]_i$ oscillation, inhibited store-operated Ca^{2+} entry, and reduced the amplitudes of Ca^{2+} responses evoked by agonists of G protein-coupled receptors. Moreover, prolonged exposure of astrocytes to AA and DHA brought the cells to a new steady state of a moderately elevated $[Ca^{2+}]_i$ level, where the cells became virtually insensitive to external stimuli. This new steady state can be considered as a mechanism of self-protection. It isolates disturbed parts of the brain, because AA and DHA reduce pathological overstimulation in the tissue surrounding the damaged area. In inflammation-related events, frequently AA and DHA exhibit opposite effects. However, in astrocytes AA and DHA exerted comparable effects on $[Ca^{2+}]_i$. Extracellularly added AA and DHA, but not EPA, were also able to induce the release of $[^3H]AA$ from prelabeled astrocytes. Therefore, we also suggest the involvement of phospholipase A_2 activation and lysophospholipid generation in the regulation of intracellular Ca^{2+} in astrocytes.

arachidonic acid / docosahexaenoic acid / eicosapentaenoic acid / prostglandins / cycloxygenase / CNS / essential fatty acid / neuroprotection

Abbreviations: AA: arachidonic acid; AACOCF$_3$: arachidyltrifluoromethylketone; cAMP: cyclic adenosine monophosphate; ATP: adenosine 5'-triphosphate; $[Ca^{2+}]_i$: concentration of free intracellular Ca^{2+}; CaM: calmodulin; CIF: calcium influx factor; CPA: cyclopiazonic acid; DHA: docosahexaenoic acid; EPA: eicosapentaenoic acid; IP$_3$: inositol 1,4,5-trisphosphate; MAPK: mitogen-activated protein kinase; PLA$_2$: phospholipase A$_2$; iPLA$_2$: calcium independent PLA$_2$, cPLA$_2$, cytosolic PLA$_2$; PUFA: polyunsaturated fatty acid; PKA: protein kinase A; SERCA: sarcoplasmic/endoplasmic-reticulum Ca^{2+}-ATPase; SOC: store-operated Ca^{2+} channel.

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1. INTRODUCTION

1.1. Polyunsaturated fatty acids, astrocytes, and brain functions

Changes in structural and functional integrity of the brain appear to correlate with alterations in membrane concentrations of polyunsaturated fatty acids (PUFA) [1]. The brain contains not only the n-6 PUFA arachidonic acid (AA), but is also highly enriched in the n-3 PUFA docosahexaenoic acid (DHA) [2]. In contrast, in peripheral tissues, phospholipids do not accumulate prominent amounts of n-3 PUFA and, therefore, cannot serve as a reservoir.

The membrane composition concerning the proportion of n-3 and n-6 PUFA in phospholipids has been shown to be crucial for normal functioning of the central nervous system (CNS) (reviewed in [3–5]). Pathophysiological conditions, such as ischemia, trauma and seizure, are associated with increased levels of free DHA and AA in the brain tissue reaching up to micromolar concentrations [6–11]. A potential neuroprotective function of polyunsaturated fatty acids for cerebral pathologies has been suggested [12, 13]. Moreover, important roles of PUFA in psychosis, schizophrenia, and creativity have been discussed [14, 15]. Hence, molecular mechanisms of action of PUFA have significant practical value.

PUFA are mainly released via activation of phospholipases A_2, which hydrolyze the sn-2 bond of cellular phospholipids generating free acids and lysophospholipids. More than 19 different isoforms of PLA_2 have been described in the mammalian system, but recent studies have focused on four major groups, namely, the group IV cytosolic PLA_2 ($cPLA_2$), the groups II and V secretory PLA_2, and the group VI Ca^{2+}-independent PLA_2 ($iPLA_2$) [16]. $cPLA_2$ is activated by elevation of the concentration of free intracellular Ca^{2+} ($[Ca^{2+}]_i$) and/or by phosphorylation by mitogen-activated protein kinase (MAPK). $cPLA_2$ preferentially hydrolyzes phospholipids containing AA and plays a key role in the biosynthesis of eicosanoids [17]. Recent evidence has established the physiological and pathological roles of the isoform $cPLA_2\alpha$. Using $cPLA_2\alpha$ knockout mice, it has been shown that this enzyme is implicated in postischemic brain injury (reviewed in [17]). Relatively little is known concerning the regulation of $iPLA_2$. Two isoforms of $iPLA_2$ derived from different genes have been described [18, 19]. All major PLA_2 isoenzymes are present in the CNS (reviewed in [20, 21]). However, their detailed role in PUFA release is still a matter of ongoing investigations.

Astrocytes, an important type of glial cells, are very active in the metabolism of PUFA [22–25]. Astrocytes, but not neurons, are capable of elongation and desaturation of essential fatty acids (linoleic and alpha-linolenic acids). For the supply of AA and DHA, neurons depend upon astrocytes [26]. Astrocytes are capable of converting AA into prostaglandins, leukotrienes and epoxygenase metabolites [22, 27] and are also sensitive to feedback regulation by prostaglandins [28]. Notably, activation of many of the metabotropic receptors in astrocytes is associated with AA release and production of prostaglandins [29–35]. We have shown in astrocytes, which were activated by different stimuli, that AA is mainly released by the Ca^{2+}-dependent PLA_2 ($cPLA_2$). However, the release of DHA which is induced simultaneously with AA is mediated by Ca^{2+}-independent PLA_2 ($iPLA_2$) sensitive to the $iPLA_2$ specific inhibitor bromoenol lactone [36]. Additionally, we have found that the release of AA and DHA is differently regulated through Ca^{2+}- and cAMP-dependent signal transduction pathways [36].

Astrocytes are able to communicate with each other and with neurons via oscillations in $[Ca^{2+}]_i$ [37, 38]. Functional coupling of cells in different tissues and organs by way of Ca^{2+} is an important mechanism for controlling and synchronizing physiological responses (for review, see [39]). During the

last few years, a great amount of evidence has been accumulated to demonstrate an active role of astrocytes in the physiology of the nervous system as dynamic regulatory elements in neurotransmission. Ca^{2+} in astrocytes is a key element for the mutual regulation of neurons and glia [40]. In addition, a plethora of receptors expressed by astrocytes is associated with G proteins that upon activation stimulate the formation of inositol 1,4,5-trisphosphate (IP$_3$), which leads to an increase of [Ca^{2+}]$_i$ through Ca^{2+} release from IP$_3$-sensitive internal Ca^{2+}stores [28, 41–45]). Our recent studies demonstrated that astrocytes acutely release both DHA and AA after activation by the neurotransmitters glutamate and bradykinin and proinflammatory substances, such as ATP and thrombin [36]. All these substances are agonists of G protein-coupled receptors involved in the regulation of Ca^{2+} signaling in astrocytes. Hence, the presence of extracellular PUFA and the conditions of intracellular PUFA release should be taken into consideration in studies of cellular Ca^{2+} signaling.

1.2. Mechanisms of regulation of Ca^{2+}

One of the most important cellular signaling pathways is a change in [Ca^{2+}]$_i$. Changes in [Ca^{2+}]$_i$ play a pivotal role in the regulation of a wide range of cellular processes [46]. Stimulation of many plasma membrane receptors by different stimuli is associated with an increase in [Ca^{2+}]$_i$. The increase of [Ca^{2+}]$_i$ is attributed to the release of Ca^{2+} from intracellular stores, first of all the endoplasmic reticulum [47], and an influx of Ca^{2+} across the plasma membrane [48]. There is evidence for the presence of two distinct mechanisms for the activation of Ca^{2+} influx by agonists in non-excitable cells, (i) a capacitative mechanism that is dependent on the depletion of intracellular Ca^{2+} stores [47, 49] and (ii) a non-capacitative mechanism whereby Ca^{2+} entry is not affected by the store content [50]. Capacitative Ca^{2+} entry, which can be readily demonstrated in almost all non-excitable

cells, is responsible for replenishing depleted intracellular stores [51, 52]. Probable candidates of channels responsible for this Ca^{2+} entry are members of the TRPC (canonical transient receptor potential) family [52]. Models for the activation of capacitative Ca^{2+} entry involve a direct interaction between plasma membrane channels and Ca^{2+} release channels on the intracellular stores or participation of a diffusible factor, the calcium influx factor (CIF) (see [51, 52] for review). A direct interaction between TRPC and two families of intracellular Ca^{2+} release channel, IP$_3$ receptors and ryanodine receptors, has been demonstrated (see [52] for review). It is becoming increasingly clear that stimulation of cells with agonists that invoke the production of Ca^{2+}-releasing messengers promotes multiple Ca^{2+} entry pathways [50, 53, 54].

In many non-excitable cells, activation of plasma membrane receptors with an agonist at low concentrations leads to [Ca^{2+}]$_i$ oscillations. Although in some instances oscillations result from the cycling of Ca^{2+} between intracellular stores and the cytoplasm [55], in most cases the transport of Ca^{2+} across the plasma membrane is crucial [56–58]. Maintenance of [Ca^{2+}]$_i$ oscillations has been explained by some models, which implicate either capacitative Ca^{2+} entry [59, 60], or non-capacitative Ca^{2+} entry [61–64]. In the scenario involving capacitative Ca^{2+}entry, this pathway is necessary for the refilling of the stores. Thus, the oscillation frequency is controlled by the refilling time [65]. Authors supporting the non-capacitative entry theory acknowledge the importance of store refilling during the response to stimuli which produce massive Ca^{2+} release from the stores, but they stress the lack of direct evidence which could confirm the notion that capacitative entry plays a role for "weak" stimuli that generate oscillations [66]. The arachidonate-regulated Ca^{2+} current, I(ARC), was suggested as a candidate for non-capacitative Ca^{2+} entry [67]. This hypothesis is very attractive due to the fact that many different stimuli induce the release of AA in various cells.

Thus, the n-6 PUFA AA, besides serving as a precursor of prostaglandins and leukotrienes, may also have an important role as a signal molecule for non-capacitative Ca^{2+} entry associated with oscillations [63, 64, 66, 68]. Other PUFA like DHA and eicosapentaenoic acid (EPA) seem to attenuate Ca^{2+} signalling in different cells. Thus, Ca^{2+} influx is suppressed by DHA in cardiac myocytes [69] and smooth muscle cells [70].

Numerous data show the beneficial effects of n-3 PUFA, first of all DHA and EPA, in many pathological conditions, in comparison to the n-6 PUFA AA [1, 71–73]. DHA might possibly attenuate the production of prostaglandins from AA by direct inhibition of cyclooxygenases. However, the action of PUFA is not restricted to the regulation of prostaglandin production. Molecular mechanisms of a direct involvement of PUFA in cell functions remain elusive. Studies of the action of n-3 and n-6 PUFA on intracellular Ca^{2+} could clarify this issue.

2. MATERIALS AND METHODS

All experimental details for establishing astrocyte cultures and measuring cytosolic Ca^{2+} by Fura-2 fluorescence are those described before [32, 74]. Briefly, primary astrocyte cell cultures were prepared from newborn rat brains and plated on round coverslips (22 mm diameter) placed in culture dishes (50 mm diameter) and incubated at 37 °C with 10% CO_2, humidified to saturation. For the experiments, the cells were used between days 7 and 12 in culture. The concentration of free intracellular Ca^{2+} ($[Ca^{2+}]_i$) was measured using the Ca^{2+} sensitive fluorescent dye Fura-2/AM. For dye loading, the cells grown on a coverslip were placed in 1 mL HEPES-buffered saline (HBS) (buffer composition in mM: 145 NaCl, 5.4 KCl, 1 $MgCl_2$, 1.8 $CaCl_2$, 25 glucose, 20 HEPES, pH 7.4 adjusted with tris(hydroxymethyl)-aminomethane)) for 30 min at 37 °C, supplemented with 2 μM Fura-2/AM. Loaded cells were transferred into a perfusion chamber with a bath volume of about 0.2 mL and mounted on an inverted microscope. During the experiments, the cells were continuously superfused with medium heated to 37 °C. For the experiments, the cells on the coverslip were chosen randomly and usually they were all the cells in the area of observation. The cells were excited alternately at 340 nm and 380 nm for 50–70 ms at each wavelength with a rate of 0.33 Hz and the resultant emission collected above 510 nm. Images were stored on a computer and subsequently the changes in fluorescence ratio (F_{340}/F_{380}) were determined from selected regions of interest covering a single cell. Fluorescence ratios from the same day measurement were compared. In part of the experiments, autofluorescence was determined at the end of each experiment and fluorescence ratios were calculated after substraction of autofluorescence and calibrated as free $[Ca^{2+}]_i$ according to a standard protocol [75].

3. RESULTS AND DISCUSSION

3.1. Involvement of polyunsaturated fatty acids (PUFA) in Ca^{2+} regulation in astrocytes

Here we attempt to give an overview of the role of PUFA in Ca^{2+} signaling in astrocytes. We have studied the influence of AA, DHA, and EPA on thrombin- [32] and ATP-induced Ca^{2+} responses [74]. We could show that the effects of AA are not mediated by its oxidative metabolites generated via 5-lipoxygenase, cyclooxygenase and cytochrome P450 monooxygenase pathways [32, 74]. Thus the effects of fatty acids themselves on Ca^{2+} regulation in rat astrocytes will be evaluated further. First, we observed that simultaneous addition of exogenous AA or DHA (10 μM) decreased the amplitude of initial Ca^{2+} responses to ATP and thrombin by 25%, both in the presence and absence of extracellular calcium (for details see the experiment testing AA in Fig. 1A). In the presence of 10 μM AA
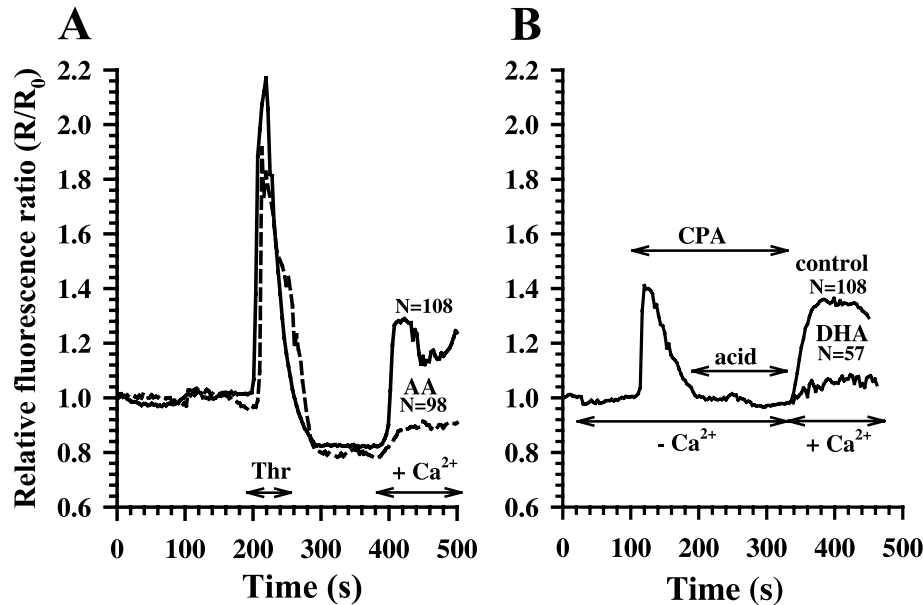
Figure 1. PUFA inhibit Ca^{2+} responses to thrombin (A) and reduce store-operated Ca^{2+} entry in astrocytes. Changes in $[Ca^{2+}]_i$ are presented as relative values (R/R_0), where R is the fluorescence ratio $(F_{340\,nm}/F_{380\,nm})$ in the cells loaded with fura-2/AM, referred to R_0, which is the R value at the beginning of the experiment. (**A**) The influence of AA on thrombin-induced Ca^{2+}release from stores and the following Ca^{2+} influx after readdition of extracellular Ca^{2+}. From the beginning of the experiment cells were superfused with Ca^{2+}-free buffer. At the time period indicated by the arrow (Thr), cells were perfused for 1 min either with 0.1 $U·mL^{-1}$ thrombin alone (solid line trace; number (N) of cells tested = 108) or for 1 min with 0.1 $U·mL^{-1}$ thrombin together with 10 μM of AA (broken line; number (N) of cells tested = 98 cells). After another 3 min, Ca^{2+} was replenished in the extracellular medium. (**B**) Influence of PUFA on Ca^{2+} entry after store depletion by SERCA inhibitor cyclopiazonic acid (CPA). Store depletion was induced by CPA (10 μM) in Ca^{2+}-free medium, followed by addition for 2 min of PUFA (acid 10 μM) as indicated by the arrows. Store-operated Ca^{2+} entry was measured as the amplitude of $[Ca^{2+}]_i$ increase after the addition of Ca^{2+} into the extracellular buffer (time period marked by $+ Ca^{2+}$). The example with the acid DHA is shown in B. Typical traces are given.

or DHA we observed a dramatic decrease of store-operated Ca^{2+} influx. The decrease was 65% and 45%, respectively. The Ca^{2+} trace example for AA is given in Figure 1A. EPA (10 μM) was again ineffective. Thus, AA and DHA can attenuate agonist-induced Ca^{2+} signals by a dual negative regulation. Firstly, there is a slight reduction of the amount of agonist-induced Ca^{2+} release from intracellular stores and secondly, there is a strong inhibition of the following Ca^{2+} influx.

We also analyzed the direct influence of AA, DHA and EPA on the capacitative Ca^{2+} influx stimulated by depletion of intracellular Ca^{2+} stores by using inhibitors of SERCA (sarcoplasmic/endoplasmic-reticulum Ca^{2+}-ATPase), such as cyclopiazonic acid (CPA) or thapsigargin, in Ca^{2+}-free medium. As shown in the example in Figure 1B, after store depletion by addition of CPA, DHA was added for 2 min and then Ca^{2+} was replenished in the extracellular medium. With application of 10 μM DHA, we observed an 80% decrease of the Ca^{2+} influx. AA (10 μM) was similarly effective. EPA (10 μM) had a weak suppressive effect
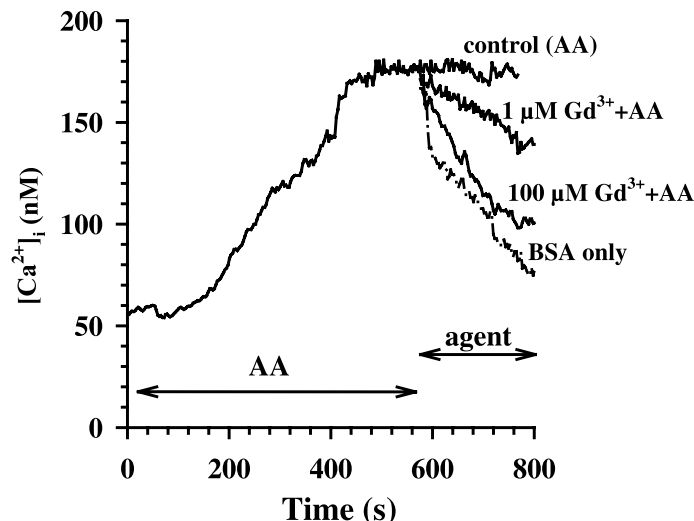
Figure 2. Induction of Ca^{2+} influx by arachidonic acid (AA) in astrocytes. Astrocytes were stimulated with 10 µM AA during the time periods indicated by the arrow marked AA. After reaching the plateau, Gd^{3+} (1 or 100 µM, number of the cells tested were 20 and 80, respectively) together with 10 µM AA, or BSA (0.1%) alone (53 cells tested) were added, during the time period indicated by an arrow marked agent. $[Ca^{2+}]_i$ was measured in the cells loaded with fura-2/AM as described in the Materials and methods. Typical traces are presented.

of less than 30% on Ca^{2+} entry after CPA-induced depletion of intracellular stores. For thapsigargin the inhibitory effect for both DHA and AA was 55%. EPA (10 µM) had no influence. SERCA-induced Ca^{2+} influx in astrocytes was reduced to a similar degree by 1 µM Gd^{3+} [74]. The lanthanide Gd^{3+} can discriminate between capacitative and non-capacitative Ca^{2+} entry pathways. Capacitative Ca^{2+} entry is sensitive to blockade by 1 µM Gd^{3+}, while non-capacitative Ca^{2+} entry is inhibited only at higher Gd^{3+} concentrations [76, 77]. Thus the channels, which are inhibited by PUFA may be attributed to store-operated Ca^{2+} channels (SOC).

Incubation of astrocytes with 10 µM AA or DHA for 30 min decreased the Ca^{2+} store content after that exposure by 55 and 40%, respectively. Ca^{2+} store content was determined by the response to CPA. EPA (10 µM) was not effective (see [32] for details). The data described in the previous paragraph suggest that the decrease in store content by

PUFA is most likely a result of the inhibition of SOC. Depletion of the Ca^{2+} stores was also observed in Ehrlich ascites tumor cells [78]. However the authors attribute this effect to Ca^{2+} release by AA from the stores.

In the absence of Ca^{2+} in the extracellular medium, no influence of PUFA on the $[Ca^{2+}]_i$ level of astrocytes was observed. This result differentiates astrocytes from several other cell systems, where AA-induced release of Ca^{2+} from stores has been shown [78–80]. In the presence of Ca^{2+} in the extracellular medium, 10 µM AA induced after prolonged incubation an increase in the $[Ca^{2+}]_i$ level by approximately 150 nM (Fig. 2). Also, the addition of DHA led in a comparable concentration-dependent manner to a delayed moderate increase in $[Ca^{2+}]_i$ (data not shown). The response displayed two distinct phases. As shown in Figure 2, after a delay of 1–2 min, a slow increase was seen reaching a new steady state after 4–5 min of application. This increase
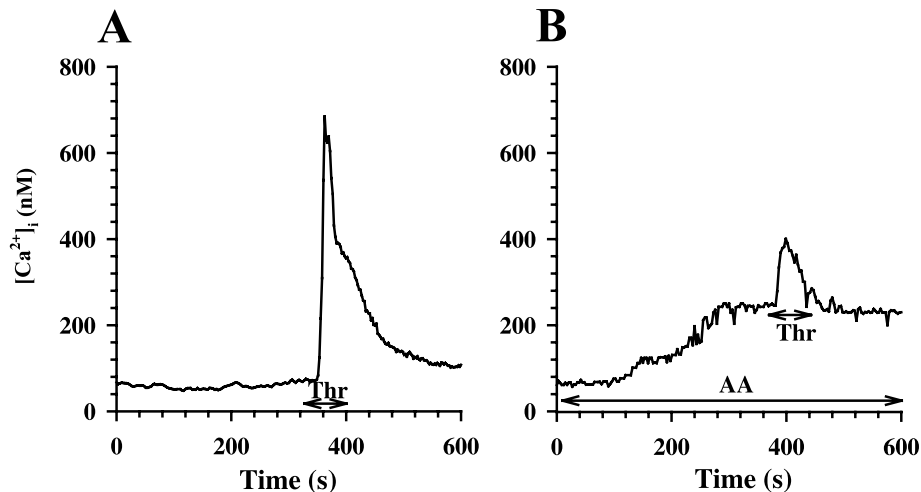
Figure 3. Prolonged incubation of rat brain astrocytes with AA dampens thrombin-induced $[Ca^{2+}]_i$ response. Control astrocytes (94 cells tested) (**A**) and astrocytes treated with 10 μM AA (23 cells tested) (**B**) were stimulated with 0.1 $U \cdot mL^{-1}$ thrombin (Thr) during the time periods indicated by the arrows. $[Ca^{2+}]_i$ was measured in the cells loaded with fura-2/AM as described in the Materials and methods. Typical traces are presented.

was reversed by the addition of Gd^{3+} [74] or by washing the cells with bovine serum albumin (0.1%), which binds and scavenges PUFA. It is important to note that 100 μM Gd^{3+} decreased $[Ca^{2+}]_i$ with a kinetics that was comparable with that observed during washing with albumin. This result indicates that AA and DHA induce $[Ca^{2+}]_i$ increase through channels which display the same sensitivity to Gd^{3+} as non-capacitative Ca^{2+} entry channels. However, when AA was added simultaneously with 1 μM Gd^{3+}, there was absolutely no increase of $[Ca^{2+}]_i$ in the astrocytes [74]. This also indicates that capacitative Ca^{2+} entry is a prerequisite for AA- and DHA- induced increase in $[Ca^{2+}]_i$.

3.2. PUFA-induced steady elevation in $[Ca^{2+}]_i$ blocks Ca^{2+} responses in astrocytes

As outlined above, AA and DHA have two opposite, but time-separated effects on intracellular Ca^{2+} in astrocytes. Firstly, AA and DHA acutely block capacitative Ca^{2+} influx. Secondly, after prolonged exposure

to AA and DHA a Ca^{2+} influx is activated that leads to the establishment of a new basal level of Ca^{2+} in astrocytes. This behavior can be described by a mathematical model of two steady states (so-called bistability), which has been developed for simulation of cellular signaling networks. This model suggests that in cellular systems, where two pathways are activated by the same stimulus, frequently both pathways are necessary to be active to reach a new steady state for a cellular parameter (in our case $[Ca^{2+}]_i$) [81, 82].

The concentration of free intracellular calcium in resting cells is the equilibrium between several influx and efflux processes. After application of 10 μM of DHA or AA and establishment of a new steady state for Ca^{2+}, the ability of astrocytes to respond to external stimuli was drastically reduced. The amplitude of the initial Ca^{2+} responses to thrombin and ATP fell from 600–800 nM (Fig. 3A) to only approximately 100 nM (Fig. 3B), as exemplified here for the response to thrombin in the presence of 10 μM AA. This may have great

physiological importance for astrocyte functions during pathological conditions, when increased extracellular levels of PUFA occur. The cells reached a new steady state level of $[Ca^{2+}]_i$, which was probably not high enough to trigger Ca^{2+}-dependent pathways. Therefore, the ability to respond to further activation by different stimuli was lost. The mechanism, which led to the reduction of responses to thrombin and ATP, is most likely the reduction of the Ca^{2+} content in cellular stores of astrocytes. A similar mechanism has been suggested for the suppression of Ca^{2+} responses caused by bradykinin and thrombin after treatment of Ehrlich ascites tumor cells with AA [78]. This suggests a widespread importance of such mechanisms for regulation of Ca^{2+} for several cell types.

In order to find further evidence for the physiological significance of elevated levels of PUFA, we investigated the influence of AA and DHA on ATP-induced $[Ca^{2+}]_i$ oscillations in astrocytes [83] and compared it with the effects of EPA (Fig. 4). Oscillations were induced by ATP and then 10 µM of PUFA was added to the perfusion medium. AA blocked the oscillations (Fig. 4A), as reported previously [32]. DHA (10 µM) also blocked the oscillations in all experiments and induced a $[Ca^{2+}]_i$ increase to a moderately elevated steady state (Fig. 4B). EPA, however, did not influence the oscillations or even increase their frequency (Fig. 4C). Thus, extracellular DHA and AA block ATP-induced $[Ca^{2+}]_i$ oscillations, which are considered to be an important part of astrocyte functions and allow astrocytes to communicate between each other and neurons [84].

The ATP-induced $[Ca^{2+}]_i$ oscillations in astrocytes were controlled by channels sensitive to Gd^{3+} at 100 µM, but not to 1 µM of Gd^{3+} (Fig. 4D). This finding allowed implying the non-capacitive type of Ca^{2+} channels in the oscillations. Studies from the Shuttleworth [85] and Taylor groups [86] have demonstrated the importance of endogenous AA in the activation of non-capaci-

tative channels and the involvement of these channels in agonist-induced $[Ca^{2+}]_i$ oscillations in some cell types. Previously, we have shown that the release of endogenous AA in astrocytes depends upon the activity of cytosolic PLA_2 [36]. Therefore, we used inhibitors of PLA_2 to investigate the possible role of endogenous AA. $AACOCF_3$, a general inhibitor of PLA_2, was employed to investigate the possible role of endogenous AA in ATP-induced oscillations. The addition of 10 µM of $AACOCF_3$ to the superfusion system 10 min before ATP or during ATP-induced $[Ca^{2+}]_i$-oscillations did not block the oscillations (data not shown). Therefore, the involvement of endogenous AA release in the regulation of $[Ca^{2+}]_i$ oscillations in astrocytes is still unclear. Experiments using different types of inhibitors of PLA_2 are required to resolve this issue.

4. CONCLUSIONS AND OUTLOOK: PROTECTIVE EFFECT OF AA AND DHA, BUT NOT EPA, ON NEURAL TISSUE

The constitutively abundant brain PUFA, AA and DHA, have various effects on different pathways of $[Ca^{2+}]_i$ regulation in astrocytes. These PUFA inhibit store-operated Ca^{2+} entry, reduce the amplitudes of responses to agonists of G protein-coupled receptors, and suppress $[Ca^{2+}]$ oscillation. Moreover, prolonged exposure of astrocytes to AA and DHA drives the cells into a new steady state with moderately elevated $[Ca^{2+}]_i$, where cells become virtually insensitive to external stimuli. We propose that this new steady state can be considered as an important part of the neuroprotective mechanisms of PUFA, because AA and DHA released by disturbed parts of the brain protect surrounding cells from pathological overstimulation.

The scheme describing the mechanism of involvement of PUFA, AA and DHA, in Ca^{2+}-triggered physiological and pathological processes in astrocytes is presented in
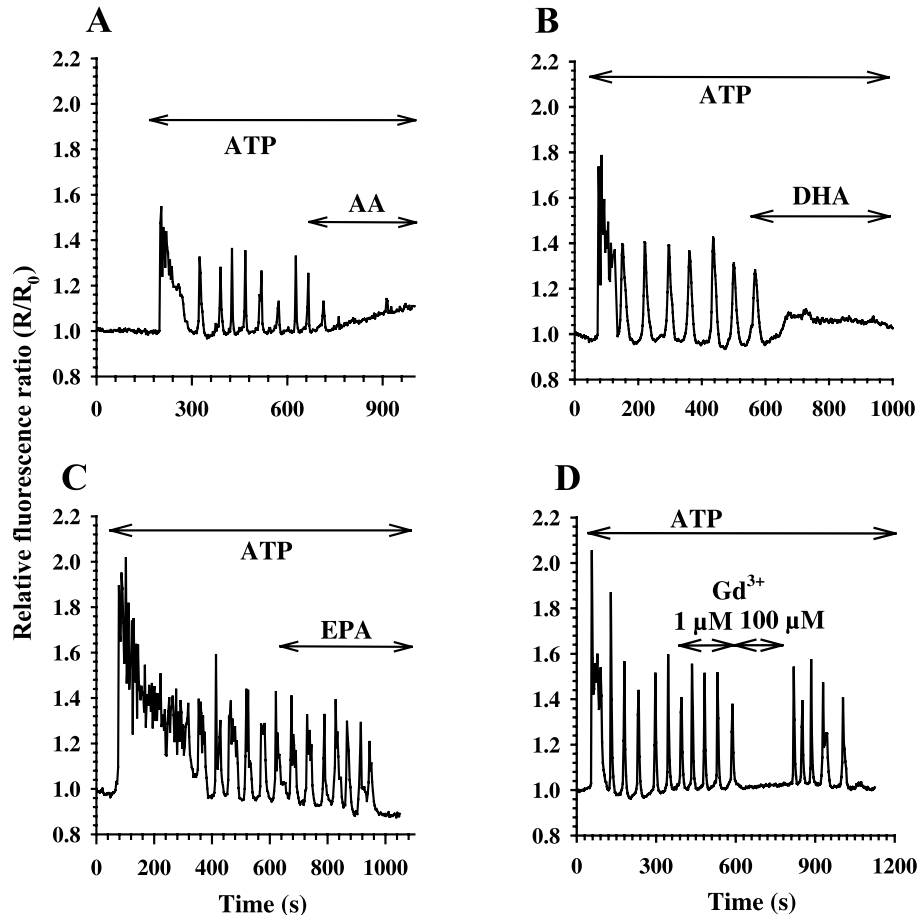
Figure 4. AA and DHA, but not EPA suppress the ATP-induced $[Ca^{2+}]_i$ oscillations in astrocytes. Fura-2-loaded cells were stimulated as indicated by arrows with ATP (10 μM) to induce $[Ca^{2+}]_i$ oscillations, before 10 μM of AA (94 cells tested) (**A**), DHA (72 cells tested) (**B**) or EPA (84 cells tested) (**C**) were added, as indicated. (**D**) Astrocytes were stimulated with ATP (10 μM) to induce $[Ca^{2+}]_i$ oscillations and then Gd^{3+} was added, as indicated by the arrows (33 cells tested). ATP-induced $[Ca^{2+}]_i$ oscillations in astrocytes were suppressed by high Gd^{3+} concentration specific for non-capacitative Ca^{2+} entry channels, but not by low concentrations of Gd^{3+} specific for capacitative Ca^{2+} channels. Typical traces are presented. R/R_0 was measured in the cells loaded with fura-2/AM, as described in Figure 1.

Figure 5. Stimulation of astrocytes by agonists induces $[Ca^{2+}]_i$ changes from the resting level of 80 nM to 500–1000 nM during spikes or oscillations. This activates different Ca^{2+} sensors, which augment a wide range of Ca^{2+}-sensitive processes. The release of small amounts of endogenous AA could participate in "on-mechanisms", which acutely decrease normal calcium responses (right part in Fig. 5). Most importantly, the excess of PUFA released outside the cells could stimulate the elevation of $[Ca^{2+}]_i$ to a new steady state of $[Ca^{2+}]_i$, which has an intermediate level of 150–200 nM. At this elevated level, the cells become insensitive to agonist stimulation; $[Ca^{2+}]_i$ spikes and
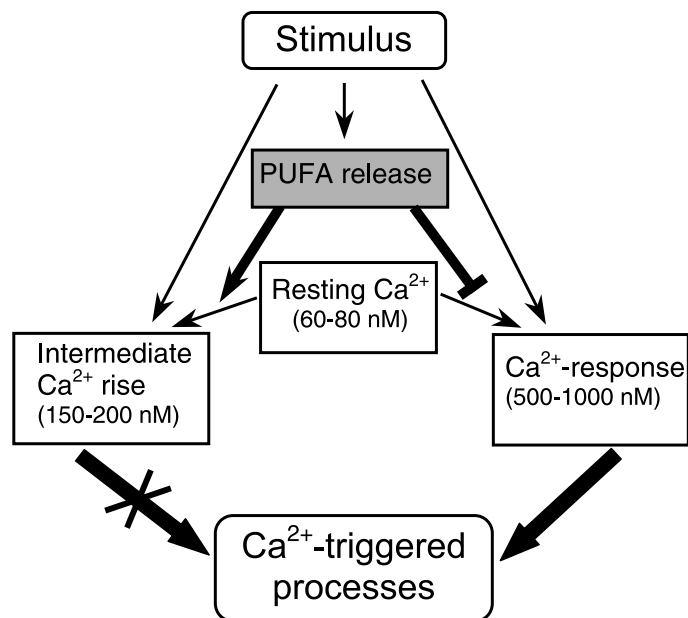
Figure 5. Pathways of suppression of cellular Ca^{2+} signaling in astrocytes by PUFA. Stimulation of astrocytes by agonists (ATP, thrombin) induces $[Ca^{2+}]_i$ change from the resting level of 80 nM to 500–1000 nM during spikes or oscillations. These signals trigger Ca^{2+}-dependent processes. PUFA which are released simultaneously, stimulate elevation of Ca^{2+} to a new steady state of $[Ca^{2+}]_i$, which has an intermediate level of 150–200 nM. At this elevated level, $[Ca^{2+}]_i$ spikes and oscillations are suppressed, whereby the Ca^{2+}-triggered processes are blocked (arrow with the cross). PUFA can also acutely decrease agonist-induced Ca^{2+}-responses (arrow with hammerhead).

oscillations are suppressed. Thus, a large part of Ca^{2+}-triggered processes is blocked (left part in Fig. 5). The Ca^{2+} system becomes less sensitive to stimulation and extracellular PUFA have a protective role.

Special attention should be given in the future to the investigation of mechanisms leading to a new steady state level of $[Ca^{2+}]_i$. Here we show that astrocytes after prolonged stimulation by AA and DHA enter into the state where they become virtually unexcitable by external stimuli which induce an increase in $[Ca^{2+}]_i$. For such bistability, a positive feedback loop is necessary [81]. PLA_2 could play a key role in the regulation. Excessive, pathological stimulation of the cells leads to a large rise in $[Ca^{2+}]_i$ and activation of other pathways, such as MAPK, PKA, which in turn activates PLA_2 and stimulates release of AA and DHA from cellular phospholipids. Then, these PUFA bring the Ca^{2+} level to the new elevated steady state and block cellular communication, which relies on $[Ca^{2+}]_i$ spikes or oscillations.

An intriguing question is still why DHA and AA differ from EPA in their action on $[Ca^{2+}]_i$. Knowledge about this question is important not only for understanding the mechanisms of $[Ca^{2+}]_i$ regulation, but also for understanding the relations between n-3 PUFA (22:6n-3 DHA and 20:5n-3 EPA) and n-6 PUFA (20:4n-6 AA). These three PUFA are all very similar in their chemical structure. The molecular mechanism, which discriminates between these fatty acid chains, is still unknown [87]. In many studies opposite effects between

these two classes of fatty acids have been observed. However, in $[Ca^{2+}]_i$ regulation in astrocytes, extracellular DHA and AA have the same effects, either positive or negative, while EPA remains ineffective.

Indeed, EPA which, in contrast to AA and DHA, failed to stimulate the release of endogenous AA from $[^3H]$-prelabeled astrocytes [32] was also inactive in the Ca^{2+} processes. Different types of PLA_2 are definitely important players in regulation of Ca^{2+} by PUFA, because endogenous release is the only significant source of free PUFA in vivo. Numerous data are now available, which provide clear evidence for the important role of PLA_2 in regulating physiological and pathological functions in the CNS [20]. PLA_2 have been implicated in the pathology of a number of neurodegenerative diseases, including Alzheimer disease, cerebral ischemia, and neuronal injury caused by excitotoxic agents (reviewed in [20]). Under normal conditions, PLA_2 isozymes may be involved in neurotransmitter release, long-term potentiation, growth and differentiation, and membrane repair. Release of AA by $cPLA_2$ may be responsible for arachidonate-regulated Ca^{2+} current [67]. Recently, an interesting pathway for the participation of $iPLA_2$ in the regulation of Ca^{2+} influx was suggested [88]. It was shown that $iPLA_2$, which in smooth muscle cells predominantly localizes to the plasma membrane, is inhibited in resting cells by calmodulin (CaM) [88]. After depletion of calcium stores, the production of a CIF, which is still unknown by nature, displaces the inhibitory CaM from $iPLA_2$, resulting in activation of $iPLA_2$ and generation of lysophospholipids, which can serve as CIF and activate SOC and capacitative calcium influx.

In the current work we focused on some aspects of the influence of PUFA on $[Ca^{2+}]_i$ in astrocytes. The involvement of PLA_2 and PUFA in the extremely complex $[Ca^{2+}]_i$ signaling network is becoming increasingly clear. Elucidation of negative and positive feedback loops between PUFA and Ca^{2+} pathways will help to understand the role of PUFA in calcium regulation in brain tissue.

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