Calcium Signals Activated by Arachidonic Acid in Embryonic Chick Ciliary Ganglion Neurons

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Key Words

Chick embryo · Arachidonic acid · Non-capacitative calcium entry · Calcium release

Abstract

Arachidonic acid (AA, 20:4) has been reported to modulate a variety of calcium-permeable ionic channels, both in the plasma membrane and in the endoplasmic reticulum. We have studied the effects of AA on calcium signaling in a well-characterized model of developing peripheral neurons, embryonic chick ciliary ganglion neurons in culture. When given at low non-micellar concentrations (5 μ M), in the majority of cells AA directly activated a delayed and long-lasting increase in $[Ca^{2+}]_i$, involving both the cytoplasm and the nucleoplasm, that was completely reversed by abolition of extracellular calcium. Other fatty acids (FAs), either saturated like arachidic acid (20:0), or unsaturated like linoleic (18:2) and docosahexaenoic acid (22:6), shared its ability to activate calcium influx. This entry was not suppressed by voltage-dependent calcium channel inhibitors ω -conotoxin and nifedipine, by the voltage-independent calcium channel antagonist LOE-908, by pre-treatment with blockers of AA metabolic pathways or with pertussis toxin. The arachidonate-activated calcium pathway was permeable to Mn^{2+} and blocked by La^{3+} , Gd^{3+} and Ni^{2+} .

In a neuronal subpopulation, AA at the same concentration was also able to elicit calcium release from thapsigargin-sensitive intracellular stores; we provide evidence that cytochrome P450 epoxygenase is involved in this process.

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Introduction

The generation of calcium signals in response to receptors for hormones, growth factors and neurotransmitters is dependent on both release of Ca^{2+} from intracellular stores and Ca^{2+} entry across the plasma membrane. Apart from the classical ionotropic receptors for neurotransmitters, channels that mediate capacitative calcium entry (CCE, or store-operated) [1] or non-capacitative calcium entry (NCCE) are not known and the mechanisms underlying their activation are still largely unclear [2]. CCE is activated as a consequence of the emptying of intracellular calcium stores; NCCE activation involves one or more second messengers linking receptor activation to channel opening. Arachidonic acid (AA), a polyunsaturated fatty acid (FA) present in the plasma membrane, has emerged as a powerful and ubiquitous intracellular messenger, linking extracellular signals to the modulation of a vast set of physiological and pathological responses.

AA is produced, following activation of membrane receptors, by different enzymes, in many cases coexisting in the same cell; in addition to acting directly on several targets, it represents the starting point of a cascade of downstream pathways, leading to a multiplicity of metabolites, collectively referred to as eicosanoids [3, 4]. AA regulates the activity of many types of ion channels including CCE and NCCE channels [5–13]. In neurons, the ability of AA to modulate voltage-dependent calcium currents has been described in detail [14–19], whereas limited information is available about its involvement in voltage-independent calcium influx pathways [20].

Here we have investigated the role of AA and its metabolism in the regulation of calcium release and calcium entry in developing chick ciliary ganglion (CG) neurons. By monitoring intracellular free calcium levels, we have found that the FA at a submicellar concentration (5 μ M) activates a delayed NCCE. In addition, we describe for the first time the ability of AA to evoke via its metabolism the release of calcium from intracellular stores in a subpopulation of CG neurons.

Materials and Methods

Cell Cultures

Chick CG neurons were obtained from 7-day embryos and maintained for 1–3 days in a chemically defined N2 medium as previously described [21]. Briefly, ganglia were incubated in divalent cation-free phosphate-buffered saline containing 0.06% trypsin at 37°C for 5 min and, after a gentle trituration, cells were resuspended in N2 medium with human recombinant bFGF (10 ng/ml, Alomone Labs, Israel). Cells were then plated in the middle area of 40-mm glass coverslips (Bioprotech, USA) or 35-mm plastic dishes (Corning, USA) coated with poly-D-lysine (100 μ g/ml) and laminin (2 μ g/cm²).

Ca²⁺ Imaging

Cells were loaded for 30 min at 37°C with 2 μ M Fluo-4 AM (Molecular Probes, USA) and were transferred in a perfusion chamber (Bioprotech, USA) and mounted on an inverted microscope (Eclipse TE 300, Nikon, Japan). Experiments were performed at a chamber temperature of 37°C. During experiments cells were continuously superfused by means of a gravity microperfusion system combined with electrovalves to allow switching between different solutions. The control solution was a standard Tyrode solution of the following composition, in mM: NaCl, 154; KCl, 4; CaCl₂, 2; MgCl₂, 1; HEPES, 5; glucose, 5.5; NaOH to pH 7.4. A calcium-free solution was obtained from the Tyrode solution by omitting CaCl₂ and adding 0.5 mM EGTA. Single-cell [Ca²⁺]_i measurements were performed exciting the dye at 494 nm for 200 ms, at a frequency of 0.2–3 Hz (according to the length of the recording), by means of a monochromator (Polychrome IV, T.I.L.L. Photonics GmbH, Germany). Images were recorded with a cooled CCD camera (SensiCam, PCO, Germany) and stored on a computer. Fluorescence

was determined from regions of interest (ROI) covering single neuronal cell bodies. Dye excitation, image acquisitions and ROI analysis protocols were performed with Axon Imaging Workbench software (Axon Instruments, USA). Estimation of fluorescence intensity was performed as the pseudoratio F/F_{base} according to the following formula: $\Delta F/F = (F - F_{\text{base}})/(F_{\text{base}} - B)$, where F is the measured fluorescence intensity of the Fluo-4 indicator, F_{base} is the fluorescence intensity of the indicator in the cell before stimulation, and B is the background signal from the averaged areas adjacent to the cell [22].

Neurons were clearly recognizable from non-neuronal cells by their rounded cell bodies and long processes: in addition, brief application of a high KCl solution (a Tyrode solution in which 40 mM of NaCl was isomolarly substituted with KCl) induced a marked and fast transient calcium increase due to influx through voltage-gated calcium channels in all morphologically identified neurons tested.

To provide an estimation of the relative amplitude of calcium signals, for each neuron, the calcium rise at the end of the stimulation (600 s) was normalized to the peak calcium increase induced by a brief (2–3 s) application of KCl in the extracellular medium prior the application of the FAs. The ratio $R = \Delta F_{\text{FA}}/\Delta F_{\text{HK}} = (F_{\text{FA}} - F_{\text{FAbase}})/(F_{\text{HK}} - F_{\text{HKbase}})$ was calculated and used to compare calcium increases elicited by various stimuli, where F_{FAbase} and F_{HKbase} are the fluorescence intensities of the indicator in the cell before HK and FA stimulation and F_{HK} and F_{FA} are the maximum values of the indicator in response to stimulations.

Fura-2 was employed to evaluate the rate of Mn²⁺ entry via AA-activated calcium influx and the effects of the metal ions Ni²⁺, Gd³⁺ and La³⁺. Cells were incubated for 30 min with 0.5 μ M Fura-2 AM (Molecular Probes, USA). For Mn²⁺ and Ni²⁺ quenching experiments, Fura-2 fluorescence was excited alternately at 360 and 380 nm and emission was recorded at 510 nm. Mn²⁺ and Ni²⁺ entry was inferred from the rate of quenching of Fura-2 fluorescence excited at the isosbestic wavelength of 360 nm when 2 mM of CaCl₂ was isomolarly substituted with either MnCl₂ or NiCl₂. Maximal values of Mn²⁺ quenching were estimated in each neuron at the end of the experiments by permeabilization of cells with 4-bromoA23187 (5 μ M). In all other Fura-2 experiments fluorescence was excited alternately at 340 and 380 nm and emission was recorded at 510 nm.

Confocal Microscopy

In order to distinguish between nuclear and cytoplasmic Ca²⁺ signals, optical sectioning was adopted. Measurements were performed using an Olympus Fluoview 200 laser scanning confocal microscope equipped with a 100 $\times$, 1.3 numerical aperture, oil-immersion objective.

Cells plated onto 35-mm coverslips were loaded with Fluo4 AM (2 μ M, 30 min, 37°C) and excited by a 488-nm, 20-mW krypton-argon ion laser. Emission signals were filtered by a 510- to 540-nm bandpass filter and detected with the scanning head. During experiments, cells were continuously bathed with the same microperfusion system described in the previous section. X–Y plane images (resolution 800 $\times$ 600 pixels) were acquired every 3 s (scan time 1.6 s), stored in the multiTIFF file format, and subsequently analyzed with ImageJ, a public domain Java image-processing software tool [Rasband W: ImageJ (Imaging Software). Version 1.31. Bethesda, National Institutes of Health, 2004]. For each experiment, a higher definition image (scan time 28 s) was collected, too,

in order to improve the identification of subcellular structures. For each image sequence, neuronal nuclear and cytoplasmic ROIs were identified and fluorescence intensity of each ROI was computed. Changes in $[Ca^{2+}]_i$ were represented by $\Delta F/F$ pseudoratio, as described previously, and plotted for the two ROIs.

Pharmacological Agents

AA (sodium salt; Sigma, USA) was dissolved in water. Stock solutions of nifedipine (Sigma, USA) were prepared daily in DMSO. ω -Conotoxin (Alomone Labs, Israel) and LOE-908 ((R,S)-(3,4-dihydro-6,7-dimethoxy-isochinolin-1-yl)-2-phenyl-N,N-di[2-(2,3,4-trimethoxyphenyl)ethyl]acetamid mesylate, Boehringer Ingelheim Pharma, Germany) were solubilized prior to the use in Tyrode solution. In some experiments, cells were incubated for at least 5 h with 500 ng/ml pertussis toxin (PTX; Sigma, USA). Nordihydroguaiaretic acid, indomethacin and econazole, linoleic acid (LA), arachidic acid (ArA), docosahexaenoic acid (DHA; Sigma, USA) thapsigargin (TG; Alomone Labs, Israel) were dissolved in DMSO.

All other reagents were obtained from Sigma (USA).

Statistical Analysis

Data are expressed as mean $\pm$ s.d. One-way ANOVA was performed to analyze experiments. Post hoc tests were used to determine where statistically significant differences were located among the groups (Tukey's test). The level of significance, unless indicated otherwise, was $p < 0.05$.

Results

AA Directly Activates a Long-Lasting Calcium Influx in CG Neurons

As a first step, we investigated the ability of AA to induce intracellular calcium signals in CG neurons. Continuous perfusion of 5 μM AA elicited in the majority of the cells tested (360 of 423) a slow and long-lasting $[Ca^{2+}]_i$ increase (fig. 1a). This effect was in general difficult to wash out and only addition of bovine serum albumin (BSA; essentially FA free, 0.5 mg/ml) in the control solution partially reversed the AA-induced calcium increase in 30 of 48 of the cells tested (fig. 1a). BSA caused a decline of Fluo-4 fluorescence that after 5 min of application was $62.8 \pm 18.3\%$. BSA binds free FAs and probably extracts AA from the lipid membrane, thus maintaining a concentration gradient for AA to diffuse from the cell into the external solution [17, 20]. On the other hand, perfusion of calcium-free medium (0 mM $CaCl_2$, 0.5 mM EGTA) completely reversed the response ($95.0 \pm 14.8\%$ of fluorescence decrease after 5 min of application) in 63 of 78 cells tested (fig. 1b), suggesting that AA had caused an influx of calcium from the extracellular medium. Neither EGTA nor BSA alone had any effect on basal $[Ca^{2+}]_i$ (fig. 1c, d, respectively).

We performed a set of experiments to test the ability of other FAs with various chain lengths and degree of saturation to elicit calcium signals in CG neurons. In separate experiments, exogenous application of DHA (fig. 1e), LA and ArA, at the same concentration used for AA (5 μM), was able to induce a calcium increase in CG neurons bathed in Tyrode standard solution. The percentage of responsive neurons was 100% for all the three FAs (LA, $n = 35$ neurons; ArA, $n = 25$, DHA, $n = 50$). As described in the Materials and Methods section, for each neuron we calculated the ratio $R = \Delta F_A / \Delta F_{HK}$, where ΔF_{HK} is the maximum increase in fluorescence induced by a brief high KCl stimulation and ΔF_A is the fluorescence increase after 600 s of FAs stimulation. We found $R_{LA} = 0.31 \pm 0.18$ (mean $\pm$ s.d.), $n = 35$ from two separate experiments; $R_{AA} = 0.15 \pm 0.08$, $n = 53$ from 6 separate experiments; $R_{DHA} = 0.21 \pm 0.11$, $n = 50$ from four separate experiments; $R_{ArA} = 0.23 \pm 0.14$, $n = 25$ from three separate experiments. These data, shown in figure 1f, suggest that other FAs are able to elicit a delayed and sustained calcium increase of greater or comparable amplitude to AA-induced signals.

A set of experiments was performed to investigate if the arachidonate-induced calcium influx could be carried through voltage-dependent calcium channels. When nifedipine (10 μM) and ω -conotoxin (2 μM) were added to the medium during the response to AA, no changes in the rate of fluorescence increase were observed in 24 of 25 cells tested: subsequent substitution of the test solution with one containing 0 $CaCl_2$, 0.5 EGTA to the external medium abolished the calcium influx (fig. 2a).

We investigated the effects of LOE-908 on the calcium influx induced by AA. The above compound has been reported to block the store-independent AA-induced calcium influx [23] as well as store-operated calcium entry [24]. When the calcium influx blocker, at a final concentration of either 20 μM (12 neurons) or 50 μM (23 neurons), was added to the external medium (Tyrode standard solution) during the response to AA, no changes in the rate of fluorescence increase were observed in all cells tested: subsequent substitution of the test solution with one containing 0 mM $CaCl_2$, 0.5 mM EGTA to the external medium abolished the calcium influx (fig. 2b). We conclude that the AA-calcium entry is insensitive to the voltage-independent calcium channel antagonist LOE-908.

In order to exclude the involvement of AA metabolites in calcium influx activation, cells were perfused for 10 min with nordihydroguaiaretic acid (20 μM), indomethacin (20 μM) and econazole (10 μM), specific in-

Fig. 1. Effect of exogenous AA and other FAs on $[Ca^{2+}]_i$. Application of 5 μM AA to the external solution induced a long lasting fluorescence increase that was reversed by BSA (**a**) and calcium-free medium (0 nominal Ca^{2+} and 0.5 mM EGTA) (**b**). **c, d** Lack of effects of BSA and calcium-free medium on $[Ca^{2+}]_i$ basal levels. **e** The sustained calcium signal obtained with DHA (5 μM) was reversed upon withdrawal of external Ca^{2+} . **f** Mean data comparing the increase in $[Ca^{2+}]_i$ elicited by various fatty acids and the calcium influx through voltage-gated calcium channels. R represents $\Delta F_A/\Delta_{HK}$ where Δ_{HK} is the maximum increase in fluorescence induced by a brief high KCl stimulation and ΔF_A is the fluorescence increase after 600 s of FAs stimulation. The number of cells is given in parentheses. ** $p < 0.001$ versus AA and DHA.

hibitors respectively of the lipoxygenase, cyclooxygenase and cytochrome P450 monooxygenase metabolic pathways, prior to stimulation with 5 μM AA. As shown in figure 2c, in these conditions AA still elicited the activation of the long-lasting calcium influx in 30 of 35 neurons, thus providing evidence that AA itself is able to activate the response.

Recently, a novel human eicosanoid receptor coupled to $G_{O/i}$ that specifically binds AA has been cloned [25]. In order to investigate if a similar receptor is involved in the response induced by the polyunsaturated FA, we performed a set of experiments after pre-incubation of cultures for at least 5 h with PTX. Under these conditions, AA was again able to activate calcium influx in 23 of 26

Fig. 2. Lack of involvement of voltage-activated calcium channels, AA metabolites, PTX-sensitive G proteins and lack of effect of LOE-908 in AA-induced calcium influx. **a** The long-lasting calcium influx was unaffected by ω -conotoxin ($2 \mu M$) and nifedipine ($10 \mu M$) and **b** LOE-908 ($20 \mu M$). **c** It was still recorded in neurons pre-incubated for at least 10 min with nordihydroguaiaretic acid (N, $20 \mu M$), indomethacin (I; $20 \mu M$) and econazole (E, $10 \mu M$) or **d** for at least 5 h with 500 ng/ml PTX.

cells (fig. 2d), thereby excluding a role for a $G_{o/i}$ coupled receptor-mediated pathway.

Finally, we used confocal microscopy to investigate the subcellular localization of the calcium increase in CG neurons. In all cells tested ($n = 9$), AA ($5 \mu M$) caused a marked increase in both cytosolic and nuclear calcium concentration, with comparable time courses. Figure 3a shows a high definition image of two cells loaded with Fluo-4: the cytosol and nucleus can easily be distinguished, allowing the definition of two ROIs. In figure 3b and c, fluorescence variations before and during the AA response are shown. Data corresponding to both ROIs are presented in figure 3d: AA induced a calcium increase in both the cytosol and the nucleus. Moreover, substitution of the test solution with one containing 0 mM $CaCl_2$,

0.5 mM EGTA to the external medium reduced the level of the response in both ROIs, confirming that the observed fluorescence increase was due to calcium influx.

At the optimal z section for observing calcium changes in both the nucleus and soma, only a few and short neuritic segments could be visualized, usually emerging from the neuronal body. In these regions calcium signals behaved as in the soma and nucleus ($n = 3$, data not shown). Because of the low temporal resolution of our confocal microscope, we were not able to detect if the AA signals originated locally in the neurites or if they resulted from the propagation of calcium elevations generated in the soma.

Fig. 3. AA induces changes in nuclear and cytoplasmic Ca^{2+} levels. **a** High definition confocal image of two CG neurons loaded with Fluo-4 that allowed the identification of nuclear and cytosolic regions. **b, c** ROI of the same area were selected at nuclear (green circle) and cytosolic (red circle) levels. **d** $[\text{Ca}^{2+}]_i$ increases in response to application of $5 \mu\text{M}$ AA to the external solution obtained from the ROIs showed in **b, c**.

Fig. 4. The calcium influx pathway activated by AA is permeable to Mn^{2+} and blocked by Ni^{2+} . **a** Comparison of Fura-2 quenching rates when 2 mM CaCl_2 was isomolarly substituted by MnCl_2 (arrow) on unstimulated (dotted line) and AA-responsive (solid line) neurons. Each trace is the average of single recordings obtained respectively from 14 and 15 neurons. The vertical bar specifies the extent of percent Mn^{2+} quench. **b** Addition of $100 \mu\text{M}$ of NiCl_2 in the external bathing solution blocks the AA-induced calcium influx. **c** Lack of effects of NiCl_2 on $[\text{Ca}^{2+}]_i$ basal levels. **d** Lack of quenching effects on Fura-2 fluorescence signal obtained a 360-nm excitation when 2 mM CaCl_2 was isomolarly substituted by NiCl_2 during the response to AA.

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Fig. 5. Effects of lanthanides on AA-calcium influx. Addition of $1 \mu\text{M}$ of LaCl_3 (**a**) or GdCl_3 (**b**) in the external bathing solution blocks the AA-calcium influx. Lack of effects of lanthanides on $[\text{Ca}^{2+}]_i$ basal levels (**c**, **d**).

Effects of Metal Ions on AA-Calcium Influx

To further characterize the activation pathway we tested the effects of divalent and trivalent ions on the AA-calcium entry in cells incubated with Fura-2 (see Methods).

A first set of experiments was performed to evaluate if Mn^{2+} permeates through the arachidonate-calcium influx pathway. For this purpose we compared the rate of Mn^{2+} transport by measuring the rate of Fura-2 quenching following equimolar substitution of CaCl_2 with MnCl_2 in unstimulated neurons and in responsive neurons after 10 min of AA perfusion. Results in figure 4a are the averages of traces obtained from 14 unstimulated neurons (dotted line) and from 15 AA-stimulated neurons (solid line). A marked increase in the quenching rate resulted in AA-responsive neurons, indicating that this pathway is permeable to Mn^{2+} . Similar results were obtained in two other sets of experiments.

Bath application of $100 \mu\text{M}$ of NiCl_2 during the response resulted in a decline in the Fura-2 ratio that after 5 min of application amounted to $45.1 \pm 13.9\%$, ($n = 16$,

fig. 4b). In order to exclude the involvement of a quenching effect of Ni^{2+} on the Fura-2 signal due to the transport of the metal ion via background or AA-activated plasma membrane channels, control experiments were performed in which 2 mM CaCl_2 was isomolarly substituted by NiCl_2 in AA-responsive neurons. No changes were observed in the 360-nm excitation fluorescence signal obtained, suggesting that little if any Ni^{2+} had entered through the influx pathway ($n = 34$; fig. 4d).

Finally either $1 \mu\text{M}$ La^{3+} or Gd^{3+} when added in separate experiments in the bath during the response to AA caused a decline in the Fura-2 ratio that after 5 min of application was $75.9 \pm 13.3\%$ ($n = 17$; fig 5a) and $58.2 \pm 18.6\%$ ($n = 12$; fig 5b) for La^{3+} and Gd^{3+} , respectively, indicating the high sensitivity of the arachidonate-calcium entry pathway to lanthanides.

Neither Ni^{2+} ($n = 15$; fig. 4c), La^{3+} ($n = 25$; fig. 5c) nor Gd^{3+} ($n = 36$; fig. 6c) alone had any effect on basal $[\text{Ca}^{2+}]_i$.

Fig. 6. AA induces a fast transient increase in intracellular calcium in a subpopulation of CG neurons. Examples of transient elevations of calcium elicited by AA stimulation in the presence (a) and in the absence (b) of extracellular calcium in the external medium.

AA Metabolism Is Involved in Calcium Release from Intracellular Stores in a Neuronal Subpopulation

When stimulated with AA, 9% of neurons (39 of 423) generated an early and transient calcium increase. Figure 6a shows a typical response obtained in a cell perfused with a control Tyrode solution. This early response was followed in the majority of cells by the slow long-lasting calcium influx described in the previous section. As shown in figure 6b the transient calcium increase was still observed in the presence of a solution containing 0 mM CaCl_2 and 0.5 mM EGTA: this result suggests that, in a subpopulation of cells (20 of 98), the polyunsaturated FA is able to induce calcium release from intracellular stores. To determine whether the source of this calcium rise was endoplasmic reticulum and/or mitochondria, we performed a set of experiments in which neurons were bathed in a solution containing 0 mM CaCl_2 , 0.5 mM EGTA and then with TG, a calcium ATP-ase inhibitor, at a final concentration of 0.1 μM for 5 min. Application of TG elicited in all neurons a slow and transient calcium increase. Subsequent stimulation with AA failed to induce any additional calcium transient in all the 73 neurons tested (from 3 different experiments; data not shown). These data suggest that endoplasmic reticulum is the intracellular store involved in the AA-induced calcium release.

The role of AA metabolic pathways was then investigated: neurons perfused for at least 10 min with a solution containing all the three inhibitors of the main metabolic pathways (nordihydroguaiaretic acid, indomethacin and

econazole, see above) were unable to generate fast calcium transients in response to AA ($n = 137$). In a subset of experiments, the three metabolic inhibitors were applied separately: stimulation with AA was able to induce calcium release in the presence of either nordihydroguaiaretic acid (15 of 117) or indomethacin (16 of 164; fig. 7a, b, respectively) but not in neurons treated with econazole ($n = 164$ cells; fig. 7c). These data suggest that cytochrome P450 epoxygenase is involved in the activation of calcium release in CG neurons.

Finally, calcium release from intracellular stores could still be evoked in PTX-treated neurons (data not shown), indicating that the activation of a $G_{o/i}$ protein-coupled receptor is not a prerequisite for this component of calcium signaling, too.

Discussion

The first relevant finding reported here is that AA induces, in CG neurons, the activation of a delayed and long-lasting calcium influx, involving an increase in the free calcium concentration in both the cytoplasm and the nucleoplasm. In all cells tested, $[\text{Ca}^{2+}]_i$ levels increased with a parallel time course in the two regions (nuclear and cytosolic), suggesting that a diffusional process of divalent cations from the cytosol to nucleus is involved. This AA-induced calcium entry in CG neurons appears to be independent of store depletion, since in the majority of

Fig. 7. AA metabolism is involved in the AA-induced calcium release from intracellular stores. In neurons perfused for at least 10 min with nordihydroguaiaretic acid (N, 20 μ M; **a**) and indomethacin (I; 20 μ M; **b**), application was still able to elicit transient elevations of intracellular calcium, but this was not the case in the presence of econazole (E, 10 μ M; **c**).

cells it was not preceded by any detectable calcium release. Moreover the response is in part, if not totally, mediated by the direct action of the unsaturated FA as indicated by its activation in neurons pre-incubated with the inhibitors of the three main metabolic pathways.

The molecular nature of calcium-permeable channels or carriers responsible for the AA-calcium influx is at present unknown and will require further investigations: several types of channels have been reported to be activated by polyunsaturated FAs, including AA (TRP channels [26, 27]; I_{ARC} channels [9]). However, we have characterized many aspects of the activation pathway for the AA-evoked calcium entry: it is partially reversed by treatment with albumin, it is permeable to Mn^{2+} , it is blocked by Ni^{2+} and it is inhibited by low concentrations (1 μ M) of Gd^{3+} and La^{3+} . Some of these features are consistent with the properties described in both mammalian central neurons and astrocytes [12, 20] for non-capacitative calcium influx evoked by AA; thus that seems to be a general mechanism for calcium entry, not restricted to a specific nervous cell population or stage of development or maturation.

We have observed that addition of 5 μ M AA to the external medium is able to induce calcium release from TG-sensitive calcium stores in a subpopulation of CG neurons, as previously described in some non-neuronal cell types but at higher concentrations of AA [28, 29]: this is one of the first reports of such signals at agonist concentrations that allow ruling out aspecific effects on the integrity of the cells. Previous studies on the ability of AA and its metabolites to regulate the activity of calcium release channels in neuronal cells have only been performed using cerebellar microsomes [30, 31]. In particular, the cerebellar intracellular calcium release channels IP_3Rs and $RyRs$, when inserted in a lipid bilayer, were found to be differently gated by AA and its metabolites: IP_3Rs were inhibited by AA whereas $RyRs$ were unaffected by this compound but fully activated by leukotriene B_4 [31]. Our data point to an AA epoxygenase metabolite(s) as a key mediator of this process: in fact the response was still evoked in the presence of lipoxygenase and cyclooxygenase inhibitors but not in cells incubated with econazole. Different P450 isoforms capable of AA metabolism are known to be expressed in brain tissue [32–34] and in particular in neurons [35]. Increasing evidence has accumulated about an involvement of cytochrome P450 epoxygenase products in the regulation of calcium homeostasis [34, 36–41], but notably our data are the first evidence of the role of these metabolites as intracellular mediators of calcium release in neurons.

Several reports have suggested an involvement of AA as a regulator of neuronal cell death or survival, neurite extension and synaptic plasticity [3, 4]. However, the exact mechanisms by which AA exerts its biological actions are still to be clarified: the observed propagation of calcium signals to the nucleus points to a possible role in the regulation of gene expression as well as in the phosphorylation of nuclear proteins, key events in the control of survival and/or differentiation [42, 43].

Finally, the observed ability of FAs other than AA to elicit a delayed calcium entry with similar extent and time course in CG neurons, points to a common mechanism of activation, that seems to be independent of the FA degree of unsaturation and length of the hydrocarbon tail. It should be noted that here we deal with the characterization of calcium signals activated by exogenous application of FAs and that their role in the physiological and

pathological processes in these cells is at present unknown. Nevertheless, DHA and AA are the major polyunsaturated FAs in the nervous system and they are preferentially generated by specific PLA₂ subtypes [44, 45]; future work aimed at clarifying the nature of the signals that control their delivery will be helpful in understanding the relationship between specific roles of these bioactive lipids and calcium signals.

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