Potentiation of Native and Recombinant P2X7-Mediated Calcium Signaling by Arachidonic Acid in Cultured Cortical Astrocytes and Human Embryonic Kidney 293 Cells

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Received October 21, 2005; accepted March 1, 2006

ABSTRACT

In the brain, arachidonic acid (AA) plays a critical role in the modulation of a broad spectrum of biological responses, including those underlying neuroinflammation. By using microfluorometry, we investigated the action of extracellular AA in the modulation of the purinoceptor P2X7-mediated elevation of [Ca^{2+}]_i, in cultured neocortical type-1 astrocytes and P2X7-, P2X2-transfected human embryonic kidney (HEK) 293 cells. We report that in cultured astrocytes, AA-induced [Ca^{2+}]_i elevation is coupled to depletion of intracellular Ca^{2+} stores and to a sustained noncapacitative Ca^{2+} entry. AA also induced a robust potentiation of the astrocytic P2X7-mediated [Ca^{2+}]_i rise evoked by the selective agonist 3'-O-(4-benzoyl)benzoyl-ATP (BzATP). Pharmacological studies demonstrate that the selective P2X7 antagonists oxidized ATP and Brilliant Blue G abrogated the AA-mediated potentiation of BzATP-evoked [Ca^{2+}]_i elevation. Fluorescent dye uptake experiments showed that the AA-induced increase in [Ca^{2+}]_i was not due to a switch of the P2X7 receptor from channel to the pore mode of gating. The synergistic effect of AA and BzATP was also observed in HEK293 cells stably expressing rat and human P2X7 but not in rat P2X2. Control HEK293 cells responded to AA exposure only with a transient [Ca^{2+}]_i elevation, whereas in those expressing the P2X7 receptor, AA elicited a potentiation of the BzATP-induced [Ca^{2+}]_i rise. Together, these findings indicate that AA mediates a complex regulation of [Ca^{2+}]_i dynamics also through P2X7-mediated Ca^{2+} entry, suggesting that variations in AA production may be relevant to the control of both the temporal and spatial kinetics of [Ca^{2+}]_i signaling in astroglial cells.

Astrocytes are involved in multiple brain functions, such as the developmental regulation of neuronal network, modulation of synaptic activity, and homeostatic control of the extracellular environment (Araque et al., 2001). Under pathological conditions, such as stroke, trauma, and seizures, or as a result of chronic neurodegenerative disorders (Alzheimer’s disease and amyotrophic lateral sclerosis), astroglial cells undergo a vigorous activation called reactive gliosis (Norenberg, 1994). Reactive astroglia acquire the ability to synthesize and secrete various cytokines and chemokines that contribute to the neuroinflammation associated with various brain diseases (Vila et al., 2001). Several lines of evidence suggest that increased extracellular level of ATP, released from damaged or dying cells, plays a pivotal role in promoting astrogliosis through stimulation of P2 purinoceptors (Franke et al., 2001). Among the different ionotropic P2X receptors described in astrocytes in situ (Franke et al., 2001), an up-regulation of P2X7 receptors was reported to be associated with astroglial reactivity observed upon ischemic brain injury (Franke et al., 2004). P2X7 is a nonselective cation channel, whereas a prolonged exposure to millimolar ATP causes the receptor to switch into a so-called pore conformation in which the channels become permeable also to large organic molecules (<900 Da) (Virginio et al., 1999). At high micromolar concentrations of ATP, P2X7 is a nonselective cation channel, whereas a prolonged exposure to millimolar ATP causes the receptor to switch into a so-called pore conformation in which the channels become permeable also to large organic molecules (<900 Da) (Virginio et al., 1999). A common feature of both conductance states is the elevation of free intracellular calcium levels ([Ca^{2+}]_i) that, as shown in immunocompetent cells (Gudipaty et al., 2003), could be critical for the biological role of this receptor. In cultured astrocytes, stimulation of P2X7 receptor induces the
expression of the chemokine monocyte chemoattractant protein-1 and transforming growth factor-β (Panenka et al., 2001). In addition, P2X7 activation causes the efflux of excitatory amino acids and purines and an increase in 2-arachidonoylglycerol production (Walter et al., 2004). Noteworthy, stimulation of P2X7 in microglia, the brain macrophages, also results in the production of various cytokines associated to neuroinflammation (Rampe et al., 2004).

The polyunsaturated fatty acid arachidonic acid (AA) is a constituent of the plasma membrane, which under various physiological and pathological conditions can be liberated and act as an autocrine and paracrine signaling molecule (Katsuki and Okuda, 1995). Increased levels of AA and its metabolites are involved in the pathogenesis of the neuroinflammation occurring as a consequence of various acute and chronic neurodegenerative disorders (Sun et al., 2004) and may play a role in cytotoxicity (Pompeia et al., 2003). Whereas the role of AA as modulator of neuronal activity is well recognized (Chalimoniuk et al., 2004), little is known about the molecular mechanisms regulated by AA in astroglial cells. High micromolar concentrations of AA were demonstrated to cause cell swelling, acidosis, and cell death of astroglial cells in vitro (Staub et al., 1994). AA also induces the closure of gap junctions in vitro (Martinez and Saez, 1999). We have demonstrated that pathophysiological concentrations of AA cause an increase in potassium (K⁺) conductance in cultured cortical astrocytes through activation of the two-pore-domain K⁺ channel TREK-2 (Ferroni et al., 2003).

It has been proposed that AA signaling may contribute to the dynamic regulation of [Ca²⁺]i in many nonexcitable cells (Watson et al., 2004). Whereas some reports have indicated that AA promotes the release of Ca²⁺ from intracellular stores (Luo et al., 2001), other studies have shown that AA-induced [Ca²⁺]i rise is associated to Ca²⁺ entry through plasma membrane capacitative Ca²⁺ entry (CCE) and non-capacitative Ca²⁺ entry (NCCE) pathways (Luo et al., 2001). In cultured astrocytes it was recently demonstrated that AA inhibits CCE and causes a delayed activation of NCCE (Sergeeva et al., 2003; Yang et al., 2005).

Because AA and P2X7 receptor play critical roles in the initiation and modulation of neuroinflammatory process, here we have explored the functional cross-talk between AA signaling and P2X7-mediated Ca²⁺ entry in cultured cortical astrocytes and upon heterologous expression of recombinant P2X7 and P2X2 in HEK293 cells. We show that AA regulates at least three Ca²⁺ entry pathways in cultured neocortical type-1 astrocytes. Micromolar concentrations of AA induce depletion of intracellular Ca²⁺ stores and activate a delayed, sustained Ca²⁺ entry pathway independent of CCE. Moreover, we found that in cultured astrocytes and only in HEK293 cells stably transfected with the P2X7 receptor, AA induces a strong potentiation of the P2X7-mediated [Ca²⁺]i rise, which is independent of AA metabolism.

**Materials and Methods**

**Cell Cultures.** Primary cultures of cortical rat astrocytes were prepared as described previously (Nobile et al., 2003) with the approval of the Committee on Animal Research of our institution. In brief, cerebral cortices of 2-day-old pups devoid of meninges were triturated and placed in cell culture flasks containing Dulbecco’s modified Eagle’s medium with 10% fetal calf serum and penicillin/streptomycin (100 U/ml and 100 μg/ml, respectively). Culture flasks were maintained in a humidified incubator at 37°C in a 5% CO₂-enriched atmosphere for 2 to 5 weeks. At confluence, astroglial cells were enzymatically dispersed (0.5-0.2 g of trypsin-EDTA) and were replated in 20-mm glass coverslips at a density of 5 x 10⁴ per coverslip. All experiments were performed at room temperature (20–22°C) at day 3 to 8 after reseeding. Immunostaining for glial fibrillary acidic protein and the flat, polygonal morphological phenotype of the cultured cells indicated that >95% were type-1 cortical astrocytes (Ferroni et al., 1995).

Cultures of the human embryonic kidney cell lines HEK293 were maintained in Dulbecco’s modified Eagle’s medium-Ham’s F-12 medium supplemented with 10% fetal bovine serum and gentamicin/glutamine (5 mg/ml and 200 mM, respectively). The plasmid containing the full-length rat P2X7-green fluorescent protein cDNA in pcDNA3 and stably human P2X7-transfected HEK293 cells were kindly provided by Dr. Di Virgilio (Department of Experimental and Diagnostic Medicine, University of Ferrara, Ferrara, Italy), whereas stably rat P2X2-transfected HEK293 cells were kindly provided by Annmarie Surprenant (Institute of Molecular Physiology, Department of Biomedical Science, University of Sheffield, Sheffield, UK).

**Stable Transfection of HEK293 Cells.** One day before transfection, cells were replated on plastic dishes (35 mm in diameter) in antibiotic-free growth medium. The following day, 4 μg of plasmid DNA was used for HEK293 transfection using cationic liposomes (Lipofectamine 2000; Invitrogen S.r.L., Milano, Italy) according to the manufacturer’s instruction. At ~16 h after transfection, the medium was changed with a medium supplemented with 1.5 g/l G418 sulfate in 100 mM HEPES.

**Immunoblotting.** The screening of the colonies for P2X7 protein was performed by Western blotting using an antibody against an intracellular domain of rat P2X7. Cells from selected clones were ruptured in lysis buffer containing 225 mM Tris, 100 mM dithiothreitol, 7% SDS, and a cocktail of protease inhibitors composed of 1 mM 4-(2-aminoethyl)benzenesulfonyl fluoride, 0.8 μM aprotinin, 0.2 μM leupeptin, 40 μM bestatin, 15 μM pepstatin, and 14 μM l-trans-epoxysuccinyl-l-leucyl amido(4-guanidino)butane. Lysates were loaded on a 10% SDS-polyacrylamide gel and blotted on a polyvinylidine difluoride membrane, blocked in 5% dry fat milk in 50 mM HEPES, and 10 mM glucose, pH adjusted to 7.4 with NaOH. The calcium-free extracellular saline was prepared by removing CaCl₂ and adding 0.5 mM EGTA. All the salts, the protease inhibitor cocktail, chemicals used for the fluorometric determinations of the [Ca²⁺]i signals, and the preparation of cultures were obtained from Sigma-Aldrich S.r.L., Milano, Italy. The Immobilon-P polyvinylidene difluoride membranes used for blotting were from Millipore (Bedford, MA), and the antibody against rat P2X7 was from Calbiochem (San Diego, CA). Lipopectamine 2000 was from Invitrogen S.r.L., and G418 sulfate was from Stratagene (La Jolla, CA). Plasmidic DNA was purified from a bacterial culture by QIAGEN Plasmid Midi kit (QIAGEN SpA, Milano, Italy). The molecular mass marker used was the wide molecular weight range from Sigma-Aldrich S.r.L. Aliquots (10 mM) of AA were prepared in dimethyl sulfoxide (DMSO) and kept at ~37°C for no longer than 1 month. On the day of experiments, perfusing solutions containing AA, the saturated fatty acids palmitic and arachidic acids, and unsaturated fatty acids oleic, linoleic, and docosahexaenoic acid (DHA) acids were prepared by diluting them in the appropriate saline and sonicating them for 5 min before use to avoid
formation of insoluble aggregates. DMSO concentration in the perfusing solution was <0.1%, and control experiments with DMSO were negative. For experiments aiming at investigating the role of AA metabolites in the observed effects, nordihydroguaiaretic acid, indomethacin, 5,8,11,13-eicosatetraynoic acid, and nicorandil (all from Sigma-Aldrich S.R.L.) were added into the extracellular bathing saline 30 min before microfluorimetric investigations.

Microfluorimetry. Intracellular calcium measurements were performed by using the fluorescent Ca2+ indicator fura-2 AM. Cells were loaded with 10 μM fura-2 AM dissolved in extracellular solution for 45 min at 37°C. The microperfusion chamber containing the cell coverslip was placed on the stage of an inverted fluorescence microscope Nikon TE2000 (Nikon, Tokyo, Japan) equipped with a dual excitation fluorometric calcium imaging system (Hamamatsu, Sunayama-Cho, Japan). Low-density seeded cells were continuously perfused at a rate of approximately 2.5 ml/min. Emission fluorescence of selected cells was passed through a narrow-band filter and acquired with a digital charge-coupled device camera (Aquadocs/In/501-S1; Hamamatsu). The sampling rate was 0.25 or 0.5 Hz. Fura-2-loaded cells were excited at 340 and 380 nm, and fluorescence was measured at 510 nm. The fluorescence ratio F340/F380 was used to monitor [Ca2+]i changes.

The changes in astrocyte permeability to the fluorescent dye 6-carboxyfluorescein (6-FAM; Invitrogen S.R.L.) were used to qualitatively explore the channel or pore mode of gating of P2X7. For these experiments, 6-FAM was added to the bathing saline in conjunction with BzATP or BzATP plus AA.

Electrophysiological Recordings. The electrophysiological studies were performed at room temperature (20–22°C) using the whole-cell configuration of the patch-clamp technique. Patch pipettes were 2 to 4 MΩ when filled with the aforesaid solutions. The external standard solution was composed of 135 mM NaCl, 5.4 mM KCl, 1 mM CaCl2, 0.1 mM MgCl2, 5 mM HEPES, and 10 mM glucose, pH adjusted to 7.4 with NaOH. The standard pipette solution contained 140 mM N-methyl-d-glucamine-Cl, 2 mM MgCl2, 5 mM TES, 5 mM EGTA, and 5 mM glucose, adjusted with N-methyl-d-glucamine+ to pH 7.3. Osmolarity was set to 316 ± 3 mOsm with mannitol. Membrane currents were recorded using an L/M EPC7 amplifier (List Electronic, Darmstadt, Germany) and were low-pass filtered at 1 kHz before acquisition. Both voltage stimulation and data acquisition were obtained using a 12-bit interface (Molecular Devices, Sunnyvale, CA) and a microcomputer equipped with pClamp, version 5.5.1 software (Axon Instruments). Voltage stimulation was performed from a holding potential of 0 mV by voltage stepping to −50 mV for 2 min.

Statistics. All data are given as means ± S.E.M. The statistical significance of differences between mean values was assessed using Student’s t test. Differences were regarded as statistically significant for P < 0.05.

Results

AA Potentiates the BzATP-Evoked [Ca2+]i Rise in Cultured Astrocytes. Calcium imaging analysis showed that exposure of cultured rat cortical type-1 astrocytes to 10 μM AA promoted a dual intracellular calcium [Ca2+]i response consisting of a Ca2+ release from intracellular stores and a sustained Ca2+ entry. As shown in Fig. 1A, in astrocytes bathed with an extracellular solution containing 1 mM Ca2+, AA caused a relatively rapid increase in [Ca2+]i, that decreased to a lower level switching to a nominally Ca2+-free solution (extracellular Ca2+ removed and addition of 0.5 mM EGTA; n = 87). A similar but slower increase in [Ca2+]i was also observed with 5 μM AA. It is noteworthy that, at variance with the exposure to 10 μM, the [Ca2+]i elevation evoked in 5 μM AA did not change in Ca2+-free saline (n = 95). These results suggest that whereas 5 μM AA evoked a [Ca2+]i rise solely because of Ca2+ release from intracellular stores, at the higher concentration an extracellular Ca2+ influx is also stimulated (Fig. 1B). Consistent with a previous report in cortical astrocytes (Sergeeva et al., 2003), the AA-induced calcium entry was depressed by low micromolar concentrations of lanthanides (La3+ and Gd3+) (data not shown).

In our recent study, we showed that challenge of cultured

![Fig. 1](image_url)

AA induces differential [Ca2+]i signals in cultured rat cortical astrocytes. A, representative traces of fluorescence ratio signal depicting the temporal kinetics of [Ca2+]i, increase in a single astrocyte loaded with the Ca2+ dye fura-2/acetoxymethyl ester. The dynamics of the [Ca2+]i response elicited by 10 μM AA in 1 mM Ca2+ and in Ca2+-free extracellular saline (0 Ca2+ plus 0.5 mM EGTA) shows the partial dependence on extracellular Ca2+ of the [Ca2+]i signal. Horizontal bars indicate the time periods of different applications. B, bar graph reporting the [Ca2+]i rise elicited by 10 μM AA and 3 μM BzATP. C, representative experiment of coapplication of 5 μM AA and 3 μM BzATP showing a sustained [Ca2+]i signal larger than that evoked by individual applications (n = 73). Dotted trace represents [Ca2+]i response obtained from the same astrocyte by subsequent application of 5 μM AA alone. Note that the AA-induced [Ca2+]i rise is slower than that evoked by coapplication of AA and BzATP. D, quantitative analysis of the synergistic action of AA and BzATP depicted by the differences in [Ca2+]i, elevations above basal levels elicited by BzATP (1–100 μM) alone (●) and BzATP in conjunction with 5 μM AA (■). Means ± S.E.M. of at least 22 cells. * P < 0.05 and ** P < 0.01, respectively.

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rat cortical type-1 astrocytes with micromolar concentrations of ATP elicited biphasic a [Ca$^{2+}$]$\text{_{i}}$ signal (Nobile et al., 2003). The initial large transient component, mediated by activation of P2 metabotropic purinoceptors (P2Y), was followed by a smaller sustained phase, principally caused by Ca$^{2+}$ influx through ionotropic P2X7-like receptor. Because the P2X7 receptor is involved in inflammatory processes so as AA (Vila et al., 2001), in this study we examined the cross-talk between the P2 signaling and AA. To test whether AA could modulate the P2X7-mediated astroglial [Ca$^{2+}$]$\text{_{i}}$ elevation, astrocytes were coexposed to different concentrations of the selective agonist BzATP and 5 μM AA, which is the concentration that induced only slow [Ca$^{2+}$]$\text{_{i}}$ responses because of Ca$^{2+}$ release from intracellular stores. Figure 1C show that whereas application of the threshold concentration of 3 μM BzATP promoted a nonsignificant [Ca$^{2+}$]$\text{_{i}}$ rise above basal level ($n = 73$; $P > 0.05$), coapplication of BzATP and AA elicited in the large majority (~80%) of the astrocytes a robust, sustained [Ca$^{2+}$]$\text{_{i}}$ elevation, which decayed to the AA-induced [Ca$^{2+}$]$\text{_{i}}$ level upon BzATP removal ($P < 0.01$). After returning to basal level, a subsequent exposure to 5 μM AA alone induced a [Ca$^{2+}$]$\text{_{i}}$ rise that was due only to Ca$^{2+}$ release from intracellular stores; this increase developed more slowly than that elicited by coapplication of AA and BzATP (Fig. 1C, dotted line). The quantitative analysis illustrates that in the range 1 to 100 μM, BzATP-mediated [Ca$^{2+}$]$\text{_{i}}$ elevation reached the steady state at approximately 10 μM. By contrast, in the presence of 5 μM AA, [Ca$^{2+}$]$\text{_{i}}$ continued to augment rather linearly up to 100 μM BzATP (Fig. 1D; $n = 22–76$). The result that the sustained [Ca$^{2+}$]$\text{_{i}}$, response generated by the coapplication of 5 μM AA and 3 μM BzATP was reversibly depressed in the presence of the broad-spectrum P2 antagonist suramin (50 μM; $n = 28$; $P < 0.01$) suggests that the potentiated [Ca$^{2+}$]$\text{_{i}}$ signal was due not to BzATP effect on AA-induced Ca$^{2+}$ influx but to AA action on the P2X7-mediated Ca$^{2+}$$\text{_{i}}$ signaling (Fig. 2A). To confirm that the P2X7 ionotropic receptor was the Ca$^{2+}$$\text{_{i}}$ entry pathway involved, experiments were performed in the presence of the selective P2X7 antagonist, oxidized ATP (oATP). After a 2-h preincubation with 100 μM oATP, 10 μM BzATP did not induce significant [Ca$^{2+}$]$\text{_{i}}$ changes. The blockage was irreversible because even upon prolonged oATP washing a coapplication of 3 μM BzATP and 5 μM AA evoked only a delayed response ascribed to the late AA-mediated [Ca$^{2+}$]$\text{_{i}}$ increase (Fig. 2B; $n = 31$). Moreover, in the continuous presence of 1 μM Brilliant Blue G, another selective inhibitor of P2X7, AA

**Fig. 2.** Synergistic action of AA and BzATP on [Ca$^{2+}$]$\text{_{i}}$ elevation involves P2X7 purinoceptor signaling. A, representative experiment showing that application of the broad-spectrum P2 purinoceptor antagonist suramin (Sura; 50 μM) caused a depression of the [Ca$^{2+}$]$\text{_{i}}$ elevation elicited by coapplication of BzATP and AA. Note that [Ca$^{2+}$]$\text{_{i}}$ decreased upon removing BzATP and returned to basal level after washout of AA ($n = 28$). B, after a 2-h astrocyte preincubation with the selective P2X7 receptor antagonist oATP (100 μM), exposure to 10 μM BzATP alone and the coapplication of 3 μM BzATP and 5 μM AA did not cause a [Ca$^{2+}$]$\text{_{i}}$, augmentation. Under this condition, the evoked [Ca$^{2+}$]$\text{_{i}}$, signal was due to the AA-dependant [Ca$^{2+}$]$\text{_{i}}$, rise as shown by the observation that BzATP removal did not significantly affect the [Ca$^{2+}$]$\text{_{i}}$, dynamics ($n = 31$). C, representative [Ca$^{2+}$]$\text{_{i}}$, response obtained by application of 0.3 μM ATP and subsequent coapplication of ATP and AA. The time period between the two stimulation was 10 min. D, histogram illustrating the [Ca$^{2+}$]$\text{_{i}}$, increases evoked by individual applications of 0.3 and 10 μM ATP or 5 μM AA, and ATP plus AA. Note that the synergistic action of AA and ATP on the sustained [Ca$^{2+}$]$\text{_{i}}$, level was depicted only upon 0.3 μM ATP. Values represent means ± S.E.M. of at least 36 cells. E, typical experiment showing the effect of 5 μM AA and 3 μM BzATP on CCE elicited by exposure to CPA in a Ca$^{2+}$-free solution to deplete the intracellular stores. F, histogram illustrating the [Ca$^{2+}$]$\text{_{i}}$, increases evoked by individual applications of BzATP alone and BzATP plus AA in presence of CPA. Values represent means ± S.E.M. of at least 33 cells.
failed to elicit BzATP-mediated [Ca$^{2+}$]$_i$ signals ($n = 14$). Together, these results demonstrate unequivocally that the potentiating effect of AA occurs only in the presence of functional P2X7 receptors.

To test whether AA was also able to modulate the biphasic [Ca$^{2+}$]$_i$ signal promoted by the endogenous purinergic agonist ATP, astrocytes were exposed to ATP concentrations ranging from 0.1 to 10 M. It is noteworthy that whereas the initial large transient component was unaffected by concomitant application of AA and ATP in the whole concentration range, a potentiation by AA of the subsequent sustained phase was depicted in the presence of ATP at the threshold concentration of 0.3 M (Fig. 2, C and D; $n = 36–72$; $P < 0.01$). It is noteworthy that with 10 M ATP, there was no potentiation by AA of the sustained [Ca$^{2+}$]$_i$ signal. Furthermore, after depletion of the intracellular Ca$^{2+}$ stores by extracellular application, in a Ca$^{2+}$-free solution, of the endoplasmic reticulum Ca$^{2+}$-ATPase inhibitor cyclopiazonic acid (CPA; 10 M), a sustained [Ca$^{2+}$]$_i$ increase mediated by store-dependent CCE pathway could be observed upon readministration of Ca$^{2+}$. Exposure to 5 M AA induced a slight decrease of the CCE-dependent steady-state [Ca$^{2+}$]$_i$ response, which increased upon coapplication of 3 M BzATP (Fig. 2, E and F; $n = 33–60$; $P < 0.01$). These findings support the notion that intracellular stores are not involved in the P2X7-mediated [Ca$^{2+}$]$_i$ potentiation by AA.

We next sought to verify whether the products of AA metabolism were involved in the modulatory action of AA on BzATP-mediated [Ca$^{2+}$]$_i$ elevation as it has previously been demonstrated in some of the AA effects on [Ca$^{2+}$]$_i$ (homeostasis depicted in various cell types (Watson et al., 2004). Thus, the next set of experiments were carried out to explore whether pharmacological interference with lipoxigenase, cyclooxygenase, and cytochrome P450 epoxygenase pathways could affect the AA-induced potentiation of the P2X7-mediated [Ca$^{2+}$]$_i$ signal. The result that in the presence of 10 M ETYA, a nonspecific blocker of all the AA-metabolizing enzymes but also a stable analog of AA (Sergeeva et al., 2003), AA continued to promote a potentiation of the BzATP-evoked [Ca$^{2+}$]$_i$ response suggests that the effect of AA is mediated either through a direct AA action or by alternative signal transduction cascades (i.e., protein kinase C and reactive oxygen species-mediated signaling) (Fig. 3A; $n = 33$). This observation was further confirmed by the finding that the AA-induced potentiation of the BzATP-evoked [Ca$^{2+}$]$_i$ signal was unchanged by the pretreatment (45 min) with specific blockers of the different pathways of AA metabolism (Fig. 3B; $n = 32–70$; $P > 0.05$). These data do not rule out the possibility that AA action in mediated through its interaction with an extracellular binding site. To address this issue, recovery from the AA action on BzATP-induced [Ca$^{2+}$]$_i$ elevation was analyzed in control conditions and in the presence of the lipid scavenger bovine serum albumin (BSA; 1 mg/ml), that, by clearing up the AA inserted in the outer leaflet of the plasma membrane, may accelerate the rate of recovery of the potentiation (Sergeeva et al., 2003). The data indicate that in the presence of extracellular BSA, the return to basal levels of the AA plus P2X7-mediated [Ca$^{2+}$]$_i$ signal upon AA removal was accelerated (Fig. 3C; $n = 25$). A similar increase in the rate of recovery upon BSA exposure was also observed on [Ca$^{2+}$]$_i$ elevation upon exposure to AA alone (data not shown). These findings strongly suggest that to potentiate the P2X7-mediated [Ca$^{2+}$]$_i$ increase, AA interacts extracellularly with some components of the plasmalemma.

Finally, the specificity of AA action was investigated by comparing the AA effect with that of other lipids with different degree of saturation and chain length of the fatty acids. The result, illustrated in Fig. 3D, clearly shows that among the various lipids tested only AA was able to generate a potentiation of the BzATP-induced [Ca$^{2+}$]$_i$ signal. This is particularly relevant for DHA, which is another major biologically active polyunsaturated fatty acid in the brain.

Fig. 3. Mechanism of action and specificity of AA effect on P2X7-mediated [Ca$^{2+}$]$_i$ increase. A, potentiating effect of AA on the BzATP-evoked [Ca$^{2+}$]$_i$ rise was not affected by the broad spectrum inhibitor of the AA metabolic pathway ETYA (10 M; $n = 33$). B, histogram depicting the lack of inhibitory actions of specific blockers of the cyclooxygenase (INDO; 10 M), lipoxigenase (NDGA; 10 M), and cytochrome P450 epoxygenase (MICO; 5 M) pathways on the [Ca$^{2+}$]$_i$ elevations induced by coapplication of 5 M AA and 3 M BzATP. Each inhibitor was administered 30 min before stimulation with AA and BzATP. Results are representative of at least 17 astrocytes for each condition. C, comparative traces showing the difference in [Ca$^{2+}$]$_i$ recovery to basal level of the P2X7-mediated [Ca$^{2+}$]$_i$ rise upon AA removal under control condition (Ctl) and in the presence of the lipid scavenger BSA (1 mg/ml). Note the increase in the rate of recovery upon BSA exposure. Superimposed to the traces are the fitting with single exponential decay. The mean time constants from various experiments were 149 ± 23 s (Ctl; $n = 23$) and 37 ± 11 s (BSA; $n = 25$). D, histogram of the effects of coapplication of 3 M BzATP with the saturated fatty acids palmitic and arachidic acids or unsaturated fatty acids oleic, linoleic, and DHA acids, all applied at a concentration of 5 M. Note that only in the presence of AA BzATP produced a significant [Ca$^{2+}$]$_i$ elevation above basal level. Columns represent the means ± S.E.M. of at least 32 cells. **, $P < 0.01$ compared with basal level.
can be released in the extracellular space and act as paracrine and autocrine signaling molecule also in astrocytes. The results reported so far indicate that AA caused a potentiation of the BzATP-induced \([\text{Ca}^{2+}]_i\) rise by both lowering the threshold of activation of P2X7 receptors and augmenting the \(\text{Ca}^{2+}\) influx; thus, we next sought to determine whether this effect could be due to a switch of the P2X7 receptor from the channel to the pore mode triggered by AA. Figure 4 shows photomicrographs of cortical astrocytes that have been incubated in a bathing medium containing the fluorescent probe 6-FAM. This molecule has a molecular weight of 376 and can be released in the extracellular space and act as paracrine and autocrine signaling molecule also in astrocytes. By contrast (PC) photomicrograph of cortical astrocyte exposed to 3 \(\mu\text{M} \) BzATP plus 5 \(\mu\text{M} \) AA caused no significant accumulation of fluorescence dye (Fig. 4, A and B). Moreover, also 30 \(\mu\text{M} \) BzATP alone did not cause an intracellular accumulation of 6-FAM (Fig. 4, C and D). By contrast, coapplication of 30 \(\mu\text{M} \) BzATP plus 5 \(\mu\text{M} \) AA induced cytoplasmic accumulation of 6-FAM often associated with membrane blebbing (Fig. 4E). Application of 300 \(\mu\text{M} \) BzATP alone in the same time period also caused an increase in cytoplasmic fluorescence (Fig. 4F). These observations suggest that the potentiating effect of AA on low BzATP-induced \([\text{Ca}^{2+}]_i\), rise is not due to an AA-mediated conformational change of P2X7 from channel to pore mode of action. After preincubation with 100 \(\mu\text{M} \) aATP, application of 300 \(\mu\text{M} \) BzATP did not induce an increase in cytoplasmic accumulation of 6-FAM fluorescence (\(n = 14\)).

**AA Potentiates the BzATP-Mediated \([\text{Ca}^{2+}]_i\), Increase in HEK293 Cells Transfected with Recombinant P2X7.** To confirm that AA positively modulates the \([\text{Ca}^{2+}]_i\), signaling specifically acting on P2X7 receptor, experiments were performed using HEK293 stably transfected with rat and human P2X7 or rat P2X2. As expected, microfluorimetric analysis performed in control HEK293 cells showed that whereas 10 \(\mu\text{M} \) ATP evoked a transient \([\text{Ca}^{2+}]_i\), response, exposure to 10 \(\mu\text{M} \) BzATP produced no \([\text{Ca}^{2+}]_i\), signal (Fig. 5A; \(n = 26\)). Application of 10 \(\mu\text{M} \) AA in Ca\(^{2+}\)-free solution induced a transient \([\text{Ca}^{2+}]_i\), response that slowly decreased to the basal level independently of readdition of extracellular Ca\(^{2+}\) (Fig. 5B), thus indicating that AA caused only Ca\(^{2+}\) release from intracellular stores. By contrast, in HEK293 cells transfected with rat P2X7 receptor (rP2X7), the threshold concentration of 1 \(\mu\text{M} \) BzATP did not affect \([\text{Ca}^{2+}]_i\), but 10 \(\mu\text{M} \) BzATP induced a large \([\text{Ca}^{2+}]_i\), rise that remained stable upon BzATP application (Fig. 5C; \(n = 65\)). This \([\text{Ca}^{2+}]_i\), response was dependent on extracellular Ca\(^{2+}\), indicating that the BzATP action required Ca\(^{2+}\) influx (data not shown).

Noteworthy, in P2X7-transfected cells, exposure to AA in Ca\(^{2+}\)-free saline induced a \([\text{Ca}^{2+}]_i\), response that was similar to that observed in nontransfected HEK293 cells (Fig. 5D; \(n = 43\)), thus strongly suggesting that the functional interaction of AA with the BzATP-induced \([\text{Ca}^{2+}]_i\), signal does not involve NCCE. To verify whether AA could potentiate the P2X7-mediated \([\text{Ca}^{2+}]_i\), rise also in transfected HEK293, cells were exposed a threshold concentration of 1 \(\mu\text{M} \) BzATP or 5 \(\mu\text{M} \) AA alone or in combination. Figure 6A shows that whereas the application of BzATP promoted only a nonsignificant \([\text{Ca}^{2+}]_i\), rise above basal level \((n = 77; P > 0.05)\), coapplication of AA and BzATP elicited a large, sustained \([\text{Ca}^{2+}]_i\), elevation, which showed a fast decay upon BzATP removal. Application of 5 \(\mu\text{M} \) AA alone induced a \([\text{Ca}^{2+}]_i\), rise that was much smaller in magnitude and developed slowly compared with the AA-modulated, BzATP-induced \([\text{Ca}^{2+}]_i\), responses. The quantitative results obtained in nontransfected and transfected HEK293 cells show unequivocally that the BzATP-evoked \([\text{Ca}^{2+}]_i\), responses in cells expressing the recombinant rP2X7 receptor are potentiated by coapplication of BzATP and AA (Fig. 6B; \(n = 35–68\)). To address the issue whether AA effect was due to an allosteric action on P2X7 receptor, inward cationic currents were recorded from rP2X7-transfected HEK293 by applying the whole-cell configuration of the patch-clamp technique on astrocytes voltage clamped to \(-50\) mV. AA (5 \(\mu\text{M} \) alone did not induce significant inward cationic currents. It is noteworthy that the currents induced by BzATP (1 and 10 \(\mu\text{M} \) did not significantly change upon coapplication of 5 \(\mu\text{M} \) AA (Fig. 6D, right; \(n = 18–24; P > 0.05\)). We next tried to determine whether such AA action was also on the human ortholog (hP2X7), which is different from the rP2X7, especially at the cytoplasmic N-terminal tail (North, 2002). Figure 6C shows that whereas the threshold concentration of 3 \(\mu\text{M} \) BzATP caused no change in \([\text{Ca}^{2+}]_i\), coapplication of 5 \(\mu\text{M} \) AA and BzATP elicited a large, sustained \([\text{Ca}^{2+}]_i\), elevation that decayed upon BzATP removal. The quantitative results obtained in hP2X7-transfected
HEK293 cells show unequivocally that also in the human ortholog, the BzATP-evoked \([\text{Ca}^{2+}]_i\) responses were potentiated by coapplication of BzATP and AA (Fig. 6D, left; \(n = 43-76; P < 0.01\)). Finally, we addressed the issue of the specificity of potentiating action of AA with respect to other members of the P2X family. BzATP-evoked \([\text{Ca}^{2+}]_i\) responses were measured in cells expressing the rat P2X2 receptor, which has some properties similar to P2X7 (North and Surprenant, 2000; North, 2002). The results indicate that coapplication of 5 \(\mu\)M AA and BzATP, at a threshold concentration of 3 \(\mu\)M, did not elicit a significant \([\text{Ca}^{2+}]_i\) elevation (\(n = 58-85; P > 0.05\)).

**Discussion**

In this study, we show that pathophysiological levels of the bioactive fatty acid AA cooperates to positively modulate the elevation of \([\text{Ca}^{2+}]_i\) mediated by stimulation of the purinergic P2X7 receptor in cultured rat astroglial cells and in HEK293 cells transfected with recombinant rat and human P2X7 recep-

![Fig. 5. Purinoceptor agonists and AA differently regulate \([\text{Ca}^{2+}]_i\) responses in HEK293 cells. A, representative control HEK293 cell showing the transient \([\text{Ca}^{2+}]_i\) rise evoked by 10 \(\mu\)M ATP and no response to 10 \(\mu\)M BzATP (\(n = 26\)). B, in the same cell, in the absence of extracellular \(\text{Ca}^{2+}\), 10 \(\mu\)M AA induced a slowly activating transient \([\text{Ca}^{2+}]_i\) increase that was not modified upon readdition of extracellular \(\text{Ca}^{2+}\). C, in HEK293 cells transfected with P2X7-green fluorescent protein, 1 \(\mu\)M BzATP did not elicit \([\text{Ca}^{2+}]_i\) increase, but subsequent application of 10 \(\mu\)M BzATP caused a robust \([\text{Ca}^{2+}]_i\) rise that rapidly returned to basal level upon BzATP removal (\(n = 65\)). D, in the same cell shown in C, in the absence of extracellular \(\text{Ca}^{2+}\), 10 \(\mu\)M AA induced a \([\text{Ca}^{2+}]_i\) response similar to that produced in nontransfected cells.

![Fig. 6. Arachidonic acid induces potentiation of BzATP-evoked \([\text{Ca}^{2+}]_i\) increase in stably transfected HEK293 cells. A, HEK293 cells expressing rP2X7 were exposed to 1 \(\mu\)M BzATP alone and in conjunction with 5 \(\mu\)M AA (\(n = 77\)). Dotted trace represents \([\text{Ca}^{2+}]_i\) response obtained from the same cell by subsequent application of 5 \(\mu\)M AA alone. B, histogram illustrating the \([\text{Ca}^{2+}]_i\) increases evoked by individual applications of BzATP or AA, and 1 \(\mu\)M BzATP plus 5 \(\mu\)M AA in nontransfected (NT) and stably transfected (T) HEK293 cells. Note the large difference in \([\text{Ca}^{2+}]_i\), elevation between P2X7-transfected HEK293 cells when stimulated with 1 \(\mu\)M BzATP or 1 \(\mu\)M BzATP plus 5 \(\mu\)M AA. Values represent means \(\pm\) S.E.M. of at least 35 cells. **, \(P < 0.01\). C, HEK293 cells expressing hP2X7 were exposed to 3 \(\mu\)M BzATP in conjunction with 5 \(\mu\)M AA (\(n = 58\)). D, left, histogram illustrating the \([\text{Ca}^{2+}]_i\) increases evoked by individual applications of BzATP or AA, and 3 \(\mu\)M BzATP plus 5 \(\mu\)M AA in hP2X7-transfected HEK293 cells. Means \(\pm\) S.E.M. of at least 43 cells. D, right, histogram illustrating the current magnitude evoked by individual applications of 1 and 10 \(\mu\)M BzATP and BzATP plus 5 \(\mu\)M AA in rP2X7-transfected HEK293 cells. Means \(\pm\) S.E.M. of at least 18 cells.
ceptors. Rat P2X2 receptors were also used to examine the specificity of AA to facilitate P2X7-mediated Ca\(^{2+}\) entry. This potentiating effect of AA is not affected by pharmacological inhibition of lipoxygenase, cyclooxygenase, and cytochrome P450 epoxygenase pathways and seems to be independent of AA-induced AA liberation through activation of the calcium-dependent cytosolic phospholipase A\(_2\) (our unpublished observations). We also confirm that high levels of exogenously applied AA stimulate a delayed Ca\(^{2+}\) entry through a non-capacitative pathway. Finally, we provide some indirect evidence that AA might potentiate Ca\(^{2+}\) entry through P2X7 in the channel mode of gating. To our knowledge, this is the first report describing the acute regulation of the P2X7 receptor-mediated [Ca\(^{2+}\)]\(_i\) response by an extracellular signaling molecule. These results are a further support to the view that signaling pathways involved in inflammatory processes can act synergistically to rapidly regulate cellular targets that play a pivotal role in the pathogenesis of neuroinflammatory diseases (Vandamme et al., 2004).

P2X7 is a subtype of the purinergic P2 class of ionotropic (X) receptor family (Franke et al., 2001) that displays a relatively low affinity for ATP (Surprenant et al., 1996). Whereas in situ the ATP~^−~ form of ATP is its endogenous ligand, it is well recognized that in vitro BzATP is a stable and potent activator of this receptor, even though it also shows a certain activity to P2X1, P2X2 and P2X3 subtypes (North and Surprenant, 2000; North, 2002). However, because Brilliant Blue G and oATP are two inhibitors of P2X7, the findings that the potentiating effect of AA on BzATP-induced [Ca\(^{2+}\)]\(_i\) signals in astroglial cells was depressed by these two agents and that AA did not influence the recombinant P2X2-mediated [Ca\(^{2+}\)]\(_i\) response strongly suggest that P2X7 was the molecular target of AA action. This result was further corroborated by the findings that a similar potentiating action of AA was observed in rat and human recombinant P2X7 receptors. We were surprised to find that the increase in [Ca\(^{2+}\)]\(_i\) levels was not paralleled by an up-regulation of P2X7-mediated current; this seems to indicate that AA does not modify the astrocytic Ca\(^{2+}\) entry by promoting an increase in Ca\(^{2+}\) permeation. However, we cannot exclude that the lack of effect of AA on BzATP-induced current was due to washout of intracellular constituents necessary for the modulatory action during the pipette dialysis. The augmentation in [Ca\(^{2+}\)]\(_i\), was not determined by an AA-mediated switch from channel to pore conformation because fluorescent dye uptake experiments depicted the pore mode in the presence of AA only at a BzATP concentration approximately 10-fold higher compared with that causing a significant potentiation of the [Ca\(^{2+}\)]\(_i\) signal. However, that AA lowered the threshold of pore formation suggests the intriguing possibility that the rise in [Ca\(^{2+}\)]\(_i\) level is causally involved in the process of pore mode of gating. Such a mechanism has been demonstrated in macrophages (Faria et al., 2005), thus indicating for the first time that a second messenger system is involved in the P2X7 pore formation.

In the brain, AA is an important signaling molecule released by receptor-stimulated phospholipase A\(_2\) in response to physiological as well as pathophysiological stimuli (Leslie, 1997). Because of its lipophilic nature, AA can act as autocrine and/or paracrine signaling molecule promoting a variety of biological effects that range from modulation of ion channels (Meves, 1994) to regulating cell growth, differentiation, and cell viability (Katsuki and Okuda, 1995). Noteworthy, AA levels are substantially up-regulated under various pathological conditions, and high micromolar levels of AA are found in brain under hypoxic/ischemic conditions, trauma, and seizures (Kinouchi et al., 1990). The role of pathological levels of free AA in the regulation of the activity of the astrocytic syncytium is still largely elusive. Indeed, although there is substantial evidence that the AA metabolites eicosanoids are potent mediators of the inflammatory process leading to the activation of astroglial cells under pathological conditions (astrogliosis) (Brambilla et al., 1999), the cellular mechanisms whereby AA signaling regulates the astrocytic reactivity are just starting to be disclosed (Brambilla and Abbracchio, 2001). Moreover, the contribution of astrogliosis in regulating the severity of neurodegeneration that develops as a result of brain insults is still controversial. In this scenario, our data showing that AA directly up-regulates the astroglial [Ca\(^{2+}\)]\(_i\), response evoked by stimulation of P2X7 purinergic receptor unravels a novel mechanism through which AA might exert its regulatory role in neurodegeneration.

In cultured astrocytes, high concentrations of AA directly modulate the intracellular [Ca\(^{2+}\)]\(_i\), rises evoked by stimulation of different purinergic receptors (Sergeeva et al., 2003), thereby indicating the ability of this signaling molecule to interact with other second messenger systems. It was also reported that AA has complex effects on the astrocytic [Ca\(^{2+}\)], dynamics by depressing CCE through inhibition of Ca\(^{2+}\) refilling of intracellular stores and by promoting a delayed, sustained NCCE Ca\(^{2+}\) entry (Sergeeva et al., 2003; Yang et al., 2005). Our data confirm and extend those findings evidencing a novel means of regulating the astroglial [Ca\(^{2+}\)], homeostasis independently to Ca\(^{2+}\) extrusion from intracellular stores. The results show that a critical determinant to elicit [Ca\(^{2+}\)], responses with different spatial and temporal kinetics is a narrow AA concentration, because 5 \(\mu\)M AA promoted a transient [Ca\(^{2+}\)], elevation because of release from intracellular stores, and concentrations above 10 \(\mu\)M AA were also able to promote a sustained Ca\(^{2+}\) influx. We also report that the potentiating effect of AA on BzATP-induced [Ca\(^{2+}\)], does not require AA metabolism. This observation is consistent with previous studies describing AA modulation of [Ca\(^{2+}\)], signals (Sergeeva et al., 2003; Yang et al., 2005) and raises a question on the molecular mechanism of AA action. Our results support the hypothesis that AA interacts extracellularly with some components of the plasma membrane because AA effect was reversed rapidly by administration of the lipid scavenger albumin. Michel et al. (2001) reported that under certain conditions BSA could reduce the potency of BzATP for P2X7 receptors but not the responses to ATP. Moreover, in the same study it was shown that AA was able to reverse the inhibitory effect of BSA on BzATP potency by a scavenging action. It is noteworthy that under our experimental conditions, a low concentration of AA was able to generate a potentiation of the astrocytic [Ca\(^{2+}\)], rise because of threshold BzATP and ATP concentrations in the absence of BSA, thereby ruling out the possibility of a scavenging effect. The reasons for these discrepancies remain to be uncovered. The result that a similar potentiating effect of AA was observed in recombinant rat and human P2X7 receptors is not surprising, because mammalian P2X7 subunits share a significant homology of approximately 80% between...
Arachidonic Acid Modulates P2X7-Mediated Calcium Response


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