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POTENTIATION OF NATIVE AND RECOMBINANT P2X7-MEDIATED CALCIUM SIGNALLING BY ARACHIDONIC ACID IN CULTURED CORTICAL ASTROCYTES AND HEK293 CELLS

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Arachidonic acid modulates P2X7-mediated calcium response

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The abbreviations used are: $[Ca^{2+}]_i$, free cytosolic Ca^{2+} concentration; AA, arachidonic acid; ATP, adenosine 5'-triphosphate; BzATP, 3'-O-(4-benzoyl)benzoyl-ATP; CCE, capacitative Ca^{2+} entry; CPA, cyclopiazonic acid; NCCE, non-capacitative Ca^{2+} entry; ETYA, 5,8,11,13-eicosatetraynoic acid; INDO, indomethacin; NDGA, nordihydroguaiaretic acid; MICO, miconazole; Sura, suramin; oATP, oxidized ATP; 6-FAM, 6-carboxyfluorescein; BSA, bovine serum albumin; BBG, brilliant blue G; DHA, docosahexaenoic acid.

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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 microfluorimetry we investigated the action of extracellular AA in the modulation of the purinoceptor P2X7-mediated elevation of free intracellular calcium ($[Ca^{2+}]_i$) in cultured neocortical type-1 astrocytes and P2X7, P2X2 transfected HEK293 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 non-capacitative 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. Altogether, 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 $[Ca^{2+}]_i$ signalling in astroglial cells.

INTRODUCTION

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 as well 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 which contribute to the neuroinflammation associated to various brain diseases (Vila et al., 2001). Several lines of evidence suggests 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 upregulation of P2X7 receptors was reported to be associated to astroglial reactivity observed upon ischemic brain injury (Franke et al., 2004). P2X7 is a ionotropic receptor characterized by a low affinity for the endogenous ligand ATP and by two states of permeability (Surprenant et al., 1996). At high micromolar concentrations of ATP, P2X7 is a non-selective 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 MCP-1 and TGF-beta (Panenka et al.,

2001). Also, P2X7 activation causes the efflux of excitatory amino acids, 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 to act as 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 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 on 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). Recently, we 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 $[Ca^{2+}]_i$ in many non-excitable cells (Watson et al., 2004). Whereas some reports have indicated that AA promotes the release of Ca^{2+} from intracellular stores (Luo et al., 2001), other studies have shown that AA-induced $[Ca^{2+}]_i$ rise is associated to Ca^{2+} entry through plasma membrane capacitative (CCE) and non-

capacitative Ca^{2+} 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).

Since AA and P2X7 receptor play critical roles in the initiation and modulation of neuroinflammatory process, here we have explored the functional crosstalk between AA signaling and P2X7-mediated Ca^{2+} 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 Ca^{2+} entry pathways in cultured neocortical type-1 astrocytes. Micromolar concentrations of AA induce depletion of intracellular Ca^{2+} stores and activates a delayed, sustained Ca^{2+} 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 $[Ca^{2+}]_i$ rise, which is independent of AA metabolism.

MATERIALS AND METHODS

Cell cultures

Primary cultures of cortical rat astrocytes were prepared as previously described (Nobile et al., 2003) with the approval of the Committee on Animal Research of our institution. Briefly, cerebral cortices of two-day-old pups devoid of meninges were triturated and placed in cell culture flasks containing Dulbecco's modified Eagle's medium with 10% foetal 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 2-5 weeks. At confluence astroglial cells were enzymatically dispersed (trypsin-EDTA, 0.5-0.2 g) and were re-plated in 20-mm glass coverslips at a density of 5 $x10^3$ per coverslip. All experiments were performed at room temperature (20-22°C) at day 3-8 after re-seeding. Immunostaining for glial fibrillary acidic protein (GFAP) 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-F12 Ham supplemented with 10% foetal bovine serum and gentamicyn/glutamine (5 $mg \cdot ml^{-1}$ and 200 mM, respectively). The plasmid containing the full length rat P2X7-GFP 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, Italy) whereas stably rat P2X2 transfected HEK293 cells were kindly provided by Annmarie Surprenant (Institute of Molecular Physiology, Dept. Biomedical Science, University of Sheffield, UK).

Stable transfection of HEK293 cells

One day before transfection cells were replated on plastic dishes (35-mm diameter) in antibiotics-free growth medium. The following day 4 µg of plasmid DNA were used for HEK293 transfection using cationic liposomes (Lipofectamine 2000) according to the manufacturer's instruction. At ~16 hours post transfection the medium was changed with one supplemented with 1.5g/l of 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 DTT, 7% SDS and a cocktail of protease inhibitors composed of: 1 mM AEBSF, 0.8 µM Aprotinin, 0.2 µM Leupeptin, 40 µM Bestatin, 15 µM Pepstatin, 14 µM E-64. Lysates were loaded on a 10% SDS Polyacrylamide gel and blotted on a PVDF membrane, blocked in 5% dry fat milk in 50 mM Tris, 150 mM NaCl and hybridised with an anti rat P2X7 polyclonal antibody. Rat cortical astrocytes were also lysed in the identical buffer and loaded on a gel with the same procedure. To address the immunospecificity of the reactions control experiments were performed using an anti rat P2X7 polyclonal antibody preabsorbed with the P2X7 antigen.

Solutions and chemicals

The extracellular bath solution contained (mM): 135 NaCl, 5.4 KCl, 1 $CaCl_2$, 0.1 $MgCl_2$, 5 Hepes and 10 glucose (pH adjusted to 7.4 with NaOH). The calcium-free extracellular saline was prepared by removing $CaCl_2$ and by adding 0.5 mM EGTA.

All the salts, the protease inihibitor cocktail, chemicals used for the fluorimetric determinations of the $[Ca^{2+}]_i$ signals and the preparation of cultures were obtained from Sigma (Sigma-Aldrich S.r.L., Italy). The Immobilon-P PVDF membranes used for blotting were from Millipore (Millipore Corporation, Bedford, MA, USA), the antibody against rat P2X7 was from Calbiochem (CN biosciences Inc., La Jolla, Ca, USA). Lipofectamine 2000 was from Gibco (Invitrogen S.r.L., Italy) and G418 Sulfate was from Stratagene (Stratagene, La Jolla, CA, USA). Plasmidic DNA was purified from a bacterial culture by Quiagen Plasmid Midi kit (Quiagen Spa, Italy). The molecular mass marker used was the Wide Molecular Weight Range from Sigma. Aliquots (10 mM) of AA were prepared in dimethylsulfuxide (DMSO) and kept at –80°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 DHA acids, were prepared by diluting them in the appropriate saline and sonicated 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 metabolic byproducts in the observed effects, nordihydroguaiaretic acid (NDGA), indomethacin (INDO), 5,8,11,13-eicosatetraynoic acid (ETYA) and miconazole (MICO) (all from Sigma) were added into the extracellular bathing saline 30 min before microfluorimetric investigations.

Microfluorimetry

Intracellular calcium measurements were performed by using the fluorescent Ca^{2+} 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

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cell coverslip was placed on the stage of an inverted fluorescence microscope Nikon TE200 (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 about 2.5 ml min^{-1}. Emission florescence of selected cells was passed through a narrow-band filter and acquired with a digital CCD camera (Hamamatsu C4742-95-12ER). Monochromator settings, chopper frequency and complete data acquisition were controlled by dedicated software (Aquacosmos/Ratio U7501-01, Hamamatsu). The sampling rate was 0.25 or 0.5 Hz. Fura-2 loaded cells were excited at 340 and 380 nm, and fluorescence measured at 510 nm. The fluorescence ratio F340/F380 was used to monitor $[Ca^{2+}]_i$ changes.

The changes in astrocyte permeability to the fluorescent dye 6-carboxyfluorescein (6-FAM; Molecular Probes, 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 2-4 MΩ when filled with the aforesaid solutions. The external standard solution was composed of (mM): 135 NaCl, 5.4 KCl, 1 $CaCl_2$, 0.1 $MgCl_2$, 5 Hepes and 10 glucose (pH adjusted to 7.4 with NaOH). The standard pipette solution contained (mM): 140 NMDG-Cl, 2 $MgCl_2$, 5 TES, 5 EGTA, 5 glucose, adjusted with $NMDG^+$ to pH 7.3. Osmolarity was set to 316±3 mOsmol with mannitol. Membrane currents were recorded using an L/M EPC7 amplifier (List Electronic, Darmstadt, Germany), and

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were low-pass filtered at 1 kHz before acquisition. Both voltage stimulation and data acquisition were obtained using a 12-bit interface (Axon Instruments, Foster City, CA) and a microcomputer equipped with pClamp (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±SEM. The statistical significance of differences between mean values was assessed using Student's *t*-test. Differences were regarded as statistically significant for $P<0.05$.

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RESULTS

AA potentiates the BzATP-evoked $[Ca^{2+}]_i$ 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 $[Ca^{2+}]_i$ response consisting of a Ca^{2+} release from intracellular stores and a sustained Ca^{2+} entry. As shown in Fig. 1A, in astrocytes bathed with an extracellular solution containing 1 mM Ca^{2+}, AA caused a relatively rapid increase in $[Ca^{2+}]_i$ that decreased to a lower level switching to a nominally Ca^{2+}-free solution (extracellular Ca^{2+} removed and addition of 0.5 mM EGTA; n=87). A similar but slower increase in $[Ca^{2+}]_i$ was also observed with 5 μM AA. Notably, at variance with the exposure to 10 μM, the $[Ca^{2+}]_i$ elevation evoked in 5 μM AA did not change in Ca^{2+}-free saline (n=95). These results suggest that whereas 5 μM AA evoked a $[Ca^{2+}]_i$ rise solely due to Ca^{2+} release from intracellular stores, at the higher concentration an extracellular Ca^{2+} 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 (La^{3+} and Gd^{3+}) (data not shown).

In our recent study we showed that challenge of cultured rat cortical type-1 astrocytes with micromolar concentrations of ATP elicited biphasic a $[Ca^{2+}]_i$ 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. Since the P2X7 receptor is involved in inflammatory processes so as AA (Vila et al., 2001), in this study we examined the crosstalk between the P2 signalling and AA. To test whether AA could modulate the P2X7-mediated astroglial $[Ca^{2+}]_i$ elevation, astrocytes were co-exposed to different concentrations of the selective agonist

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BzATP and 5 μM AA, which is the concentration that induced only slow $[Ca^{2+}]_i$ responses due to Ca^{2+} release from intracellular stores. Figure 1C show that whereas application of the threshold concentration of 3 μM BzATP promoted a non-significant $[Ca^{2+}]_i$ rise above basal level (n=73; *P*>0.05), co-application of BzATP and AA elicited in the large majority (~80%) of the astrocytes a robust, sustained $[Ca^{2+}]_i$ elevation, which decayed to the AA-induced $[Ca^{2+}]_i$ level upon BzATP removal (*P*<0.01). Following the returning to basal level, a subsequent exposure to 5 μM AA alone induced a $[Ca^{2+}]_i$ rise, only due to Ca^{2+} release from intracellular stores, that developed slower than that elicited by co-application of AA and BzATP (Fig. 1C, dotted line). The quantitative analysis illustrates that in the range 1-100 μM, BzATP-mediated $[Ca^{2+}]_i$ elevation reached the steady-state at about 10 μM. By contrast, in the presence of 5 μM AA, $[Ca^{2+}]_i$ continued to augment rather linearly up to 100 μM BzATP (Fig. 1D; n=22-76). The result that the sustained $[Ca^{2+}]_i$ response generated by the co-application of AA (5 μM) and Bz-ATP (3 μM) was reversibly depressed in the presence of the broad spectrum P2 antagonist suramin (Sura; 50 μM; n=28; *P*<0.01) suggests that the potentiated $[Ca^{2+}]_i$ signal was not due to BzATP effect on AA-induced Ca^{2+} entry but due to AA action on the P2X7-mediated Ca^{2+} signalling (Fig. 2A). To confirm that the P2X7 ionotropic receptor was the Ca^{2+} 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, BzATP (10 μM) did not induce significant $[Ca^{2+}]_i$ changes. The blockage was irreversible as even upon prolonged oATP washing a co-application of BzATP (3 μM) and AA (5 μM) only evoked a delayed response ascribed to the late AA-mediated $[Ca^{2+}]_i$ increase (Fig. 2B; n=31). Moreover, in the continuous presence of brilliant

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blue G (BBG, 1 μM), another selective inhibitor of P2X7, AA failed to elicit BzATP-mediated $[Ca^{2+}]_i$ signals (n=14). Altogether, 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 μM. Interestingly, 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 μM (Fig. 2C,D; n=36-72; $P<0.01$). Notably, with 10 μM 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 μM), a sustained $[Ca^{2+}]_i$ increase mediated by store-dependent capacitative Ca^{2+} entry (CCE) pathway could be observed upon re-addition of Ca^{2+}. Exposure to 5 μM AA induced a slight decrease of the CCE-dependent steady-state $[Ca^{2+}]_i$ response which increased upon co-application of 3 μM BzATP (Fig. 2E,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

lipoxygenase, cyclo-oxygenase 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 μM ETYA, a non-specific blocker of all the AA-metabolizing enzymes but also a stable analogue 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 signalling) (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 $mg \cdot ml^{-1}$), 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

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the fatty acids. The result illustrated in Figure 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 docosahexaenoic acid (DHA), which is another major biologically active polyunsaturated fatty acid in the brain that can be released in the extracellular space and act as paracrine and autocrine signalling molecule also in astrocytes. The results reported so far indicate that AA caused a potentiation of the BzATP-induced $[Ca^{2+}]i$ rise by both lowering the thereshold of activation of P2X7 receptors and augmenting the Ca2+ influx, and thus we next asked whether this effect could be due to a switch of the P2X7 receptor from the channel to the pore mode triggered by AA. In Figure 4 are shown photomicrographs of cortical astrocytes that have been incubated in a bathing medium containing the fluorescent probe 6-carboxyfluorescein (6-FAM). This molecule has a molecular weight of 376, and thus can pass through P2X7 receptor only when the receptor is in the pore mode (Deuchars et al., 2001). Visual analysis of the fluorescence loading indicates that 5 min co-application of 40 μM 6-FAM and 3 μM BzATP *plus* 5 μM AA did not cause any significant accumulation of fluorescence dye (Fig. 4A,B). Moreover, also 30 μM BzATP alone did not cause an intracellular accumulation of 6-FAM (Fig. 4C,D), By contrast, co-application of 30 μM BzATP *plus* 5 μM AA induced cytoplasmic accumulation of 6-FAM often associated to membrane blebbing (Fig. 4E). Application of 300 μM 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 $[Ca^{2+}]i$ rise is not due to an AA-mediated conformational change of P2X7 from channel to pore mode of action. After preincubation with 100 μM oATP, application of 300 μM BzATP did not induce an increase in cytoplasmic accumulation of 6-FAM fluorescence (n=14).

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AA potentiates the BzATP-mediated $[Ca^{2+}]_i$ increase in HEK293 cells transfected with recombinant P2X7

To confirm that AA positively modulates the $[Ca^{2+}]_i$ signalling 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 μM ATP evoked a transient $[Ca^{2+}]_i$ response, exposure to 10 μM BzATP did not produce any $[Ca^{2+}]_i$ signal (Fig. 5A; n=26). Application of 10 μM AA in Ca^{2+}-free solution induced a transient $[Ca^{2+}]_i$ response that slowly decreased to the basal level independently of re-addition 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), whereas the threshold concentration of 1 μM BzATP did not affect $[Ca^{2+}]_i$, 10 μM BzATP induced a large $[Ca^{2+}]_i$ rise that remained stable upon BzATP application (Fig. 5C; n=65). This $[Ca^{2+}]_i$ 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 $[Ca^{2+}]_i$ response that was similar to that observed in non-transfected HEK293 cells (Fig. 5D, n=43), thus strongly suggesting that the functional interaction of AA with the BzATP-induced $[Ca^{2+}]_i$ signal does not involve NCCE. To verify whether AA could potentiate the P2X7-mediated $[Ca^{2+}]_i$ rise also in transfected HEK293, cells were exposed a threshold concentration of BzATP (1 μM) or AA (5 μM) alone or in combination. Figure 6A shows that whereas the application of BzATP promoted only a non-significant $[Ca^{2+}]_i$ rise above basal level (n=77; $P>0.05$), co-application of AA and BzATP elicited a large, sustained $[Ca^{2+}]_i$ elevation, which showed a fast decay

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upon BzATP removal. Application of 5 μM AA alone induced a $[Ca^{2+}]_i$ rise that was much smaller in magnitude and developed slowly compared to the AA-modulated, BzATP-induced $[Ca^{2+}]_i$ responses. The quantitative results obtained in non-transfected (NT) and transfected HEK293 (T) cells show unequivocally that the BzATP-evoked $[Ca^{2+}]_i$ responses in cells expressing the recombinant rP2X7 receptor are potentiated by co-application 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 μM) alone did not induce significant inward cationic currents. Interestingly, the currents induced by BzATP (1, 10 μM) did not significantly change upon co-application of 5 μM AA (Fig. 6D,*right*; n=18-24; $P>0.05$). We next asked whether such AA action was also on the human orthologue (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 μM BzATP did not cause any change in $[Ca^{2+}]_i$,co-application of AA (5 μM) and BzATP elicited a large, sustained $[Ca^{2+}]_i$ elevation, which decayed upon BzATP removal. The quantitative results obtained in hP2X7-transfected HEK293 cells show unequivocally that also in the human orthologue, the BzATP-evoked $[Ca^{2+}]_i$ responses were potentiated by co-application 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 $[Ca^{2+}]_i$ 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 co-application of AA (5

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μM) and BzATP, at a threshold concentration of 3 μM, did not elicit a significant $[Ca^{2+}]_i$ elevation (n=58-85; *P*>0.05).

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DISCUSSION

In this study we show that pathophysiological levels of the bioactive fatty acid AA co-operates to positively modulate the elevation of $[Ca^{2+}]_i$ mediated by stimulation of the purinergic P2X7 receptor in cultured rat astroglial cells and in HEK293 cells transfected with recombinant rat and human P2X7 receptors. 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, cyclo-oxygenase, and cytochrome P 450 epoxygenase pathways and appears to be independent of AA-induced AA liberation through activation of the calcium-dependent cytosolic phospholipase A_2 (our unpublished observation). We also confirm that high levels of exogenously applied AA stimulates a delayed Ca^{2+} entry through a non-capacitative pathway. Finally, we provide some indirect evidence that the AA might potentiate the 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 signalling molecule. These results are a further support to the view that signalling 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, since brillant blue G and oATP are two

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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 suggests 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. Surprisingly, the fact that the increase in $[Ca^{2+}]_i$ levels was not paralleled by an up-regulation of P2X7-mediated current 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 wash-out of intracellular constituents necessary for the modulatory action during the pipette dialysis. The augment 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 about 10-fold higher compared to that causing a significant potentiation of the $[Ca^{2+}]_i$ signal. However, the fact that AA lowered the threshold of pore formation suggest the intriguing possibility that the rise in $[Ca^{2+}]_i$ level is causally involved in the process of pore mode of gating. Recently, such a mechanism was 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 signalling molecule released by receptor-stimulated phospholipase A_2 in response to physiological as well as pathophysiological stimuli (Leslie, 1997). Because of its lipophylic nature, AA can act as autocrine and/or paracrine signalling molecule promoting a variety of biological effects that range from modulation of ion channels (Meves, 1994) to regulating cell

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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. In fact, 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 signalling regulate the astrocytic reactivity are just starting to be disclosed (Brambilla and Abbracchio, 2001). Moreover, the contribution of astrogliosis in regulating the severity of neurodegeneration which 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 metabotrobic 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 $[Ca^{2+}]_i$ dynamics by depressing CCE through inhibition of Ca2+ refilling of intracellular stores, and promoting a delayed, sustained NCCE Ca2+ entry (Sergeeva et al., 2003; Yang et al., 2005). Our data confirm and extend those findings evidencing a novel means of regulating the astroglial $[Ca^{2+}]_i$ homeostasis independently to Ca^{2+} extrusion from intracellular stores. The results show that a critical determinant to elicit $[Ca^{2+}]_i$ responses with different spatial and

temporal kinetics is a narrow AA concentration, as 5 µM AA promoted a transient $[Ca^{2+}]_i$ elevation due to release from intracellular stores, concentrations above 10 µM of AA were also able to promote a sustained Ca^{2+} influx. We also report that the potentiating effect of AA on BzATP-induced $[Ca^{2+}]_i$ does not require AA metabolism. This observation is consistent with previous studies describing AA modulation of $[Ca^{2+}]_i$ signals (Sergeeva et al., 2003; Yang et al., 2005) and rises the 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. Notably, under our experimental conditions, a low concentration of AA was able to generate a potentiation of the astrocytic $[Ca^{2+}]_i$ rise due to 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 about 80% between rat and human (Chessell et al., 1998). In this context, site-directed mutagenesis studies shall provide more mechanistic insights into this AA action. The finding that qualitatively similar results were obtained in cultured astrocytes and P2X7 heterologously expressed also rule out the possibility that the potentiating action of AA in cultured astroglial cells was just due to an

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increase in electrical driving force for Ca2+ entry because of the concomitant activation by AA of a hyperpolarizing conductance such as the TREK potassium channel (Ferroni et al., 2003).

Another important issue that still remains to be addressed is the biological significance of these results. In the brain, P2X7 receptor has been proposed to be part of the signalling cascade leading to neuroinflammation and neurodegeneration (Franke et al., 2004; Wang et al., 2004). How activation of astrocytic P2X7 receptor contributes to the development of these processes still remains to be unequivocally established. There is accumulating in vitro evidence that in astrocytes (Panenka et al., 2001) as well as in microglial cells (Rampe et al., 2004), P2X7 activation promotes the synthesis and release of various cytokines and chemokines that may co-operate to regulate the inflammatory and degenerative responses, although some recent in vivo data have challenged this view (Le Feuvre et al., 2003). Given the importance of $[Ca^{2+}]$i elevation in the biochemical cascades leading to apoptotic cell death (Franklin and Johnson, 1992), and because of the correlation of P2X7-mediated cell death with the pore configuration of the receptor (Virginio et al., 1999), it is tempting to speculate that the ability of AA to potentiate the P2X7-mediated $[Ca^{2+}]_i$ rise by promoting the formation of P2X7 pore would induce the apoptotic cell death of astroglial cells. Further studies are warranted to clarify whether the synergistic action of BzATP and AA on $[Ca^{2+}]$i affects astrocyte viability.

In summary, the data reported in this study about the synergistic action of AA and ATP in the regulation of [Ca2+]i are a further support to the tenet that P2X7-mediated signalling may be a crucial therapeutic target to combat neurodegenerative

diseases and provide novel evidence on possible sites of pharmacological intervention.

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FOOTNOTES

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FIGURE LEGENDS

Fig. 1. AA induces differential $[Ca^{2+}]_i$ signals in cultured rat cortical astrocytes. A) Representative traces of fluorescence ratio signal depicting the temporal kinetics of $[Ca^{2+}]_i$ increase in a single astrocyte loaded with the Ca^{2+} dye fura-2/AM. The dynamics of the $[Ca^{2+}]_i$ response elicited by 10 μM AA in 1 mM Ca^{2+} and in Ca^{2+}-free extracellular saline (0 Ca^{2+}+ 0.5 mM EGTA) shows the partial dependence on extracellular Ca^{2+} of the $[Ca^{2+}]_i$ signal. Horizontal bars indicate the time periods of different applications. B) The bar graph reporting the $[Ca^{2+}]_i$ rises above basal levels evoked by 5 and 10 μM AA in the presence or absence of extracellular Ca^{2+} denotes that upon removal of extracellular Ca^{2+} the steady-state $[Ca^{2+}]_i$ signal elicited by 10 μM AA was significantly reduced. Values represent the means±SEM of at least 55 cells. C) Representative experiment of co-application of AA (5 μM) and BzATP (3 μM) showing a sustained $[Ca^{2+}]_i$ signal larger than that evoked by individual applications (n=73). Dotted trace represents $[Ca^{2+}]_i$ response obtained from the same astrocyte by subsequent application of 5 μM AA alone. Note that the AA induced $[Ca^{2+}]_i$ rise is slower than that evoked by co-application of AA and BzATP. D) Quantitative analysis of the synergistic action of AA and BzATP depicted by the differences in $[Ca^{2+}]_i$ elevations above basal levels elicited by BzATP (1-100 μM) alone (●) and BzATP in conjunction with 5 μM AA (■). Means±SEM of at least 22 cells. Single and double asterisks denote $p<0.05$ and $p<0.01$, respectively.

Fig. 2. The synergistic action of AA and BzATP on $[Ca^{2+}]_i$ 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 $[Ca^{2+}]_i$ elevation elicited by co-application of BzATP and AA. Note that $[Ca^{2+}]_i$ decreased upon removing BzATP and returned to basal level after wash out of AA (n=28). B) After a 2-h astrocyte preincubation with the selective P2X7 receptor antagonist oxidized ATP (oATP, 100 μM), exposure to 10 μM BzATP alone and the co-application of BzATP (3 μM) and AA (5 μM) did not cause a $[Ca^{2+}]_i$ augment. Under this condition, the evoked $[Ca^{2+}]_i$ signal was due to the AA-dependent $[Ca^{2+}]_i$ rise as shown by the observation that BzATP removal did not significantly affect the $[Ca^{2+}]_i$ dynamics (n=31). C) Representative $[Ca^{2+}]_i$ response obtained by application of ATP (0.3 μM) and subsequent co-application of ATP and AA. The time period between the two stimulation was 10 min. D) Histogram illustrating the $[Ca^{2+}]_i$ increases evoked by individual applications of ATP (0.3, 10 μM) or AA (5 μM), and ATP *plus* AA. Note that the synergistic action of AA and ATP on the sustained $[Ca^{2+}]_i$ level was depicted only upon 0.3 μM ATP. Values represent means±SEM of at least 36 cells. E) Typical experiment showing the effect of AA (5 μM) and BzATP (3 μM) on CCE elicited by exposure to CPA in a Ca^{2+}-free solution to deplete the intracellular stores. F) Histogram illustrating the $[Ca^{2+}]_i$ increases evoked by individual applications of BzATP alone and BzATP *plus* AA in presence of CPA. Values represent means±SEM of at least 33 cells.

Fig. 3. Mechanism of action and specificity of AA effect on P2X7-mediated $[Ca^{2+}]_i$ increase. A) The 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 μM; n=33). B) Histogram depicting the lack of inhibitory actions of specific blockers of the

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cyclo-oxygenase (INDO, 10 μM), lipoxygenase (NDGA, 10 μM) and cytochrome P450 epoxygenase (MICO, 5 μM) pathways on the $[Ca^{2+}]_i$ elevations induced by co-application of AA (5 μM) *and* BzATP (3 μM). 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 bovine serum albumin (BSA; 1 $mg \cdot ml^{-1}$). 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 co-application of BzATP (3 μM) 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 μM. Note that only in the presence of AA BzATP produced a significant $[Ca^{2+}]_i$ elevation above basal level. Bars represent the means±SEM of at least 32 cells. Double asterisk denotes $p< 0.01$ compared to basal level.

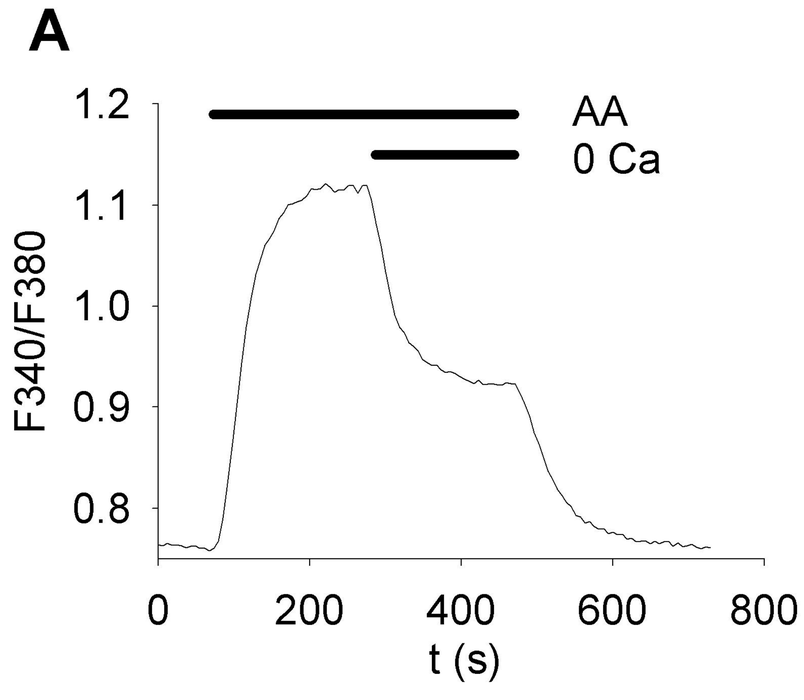
Fig. 4. The synergistic action of 3 μM BzATP and 5 μM AA on $[Ca^{2+}]_i$ rise is not mediated through the pore mode of gating of P2X7 receptor. A) Phase-contrast (PC) photomicrograph of cortical astrocyte exposed to BzATP (3 μM) *plus* AA (5 μM) for 5 min in the presence of the fluorescence probe 6-carboxyfluorescein (6-FAM; 40 μM). B) The same astrocyte viewed under fluorescence illumination does not show

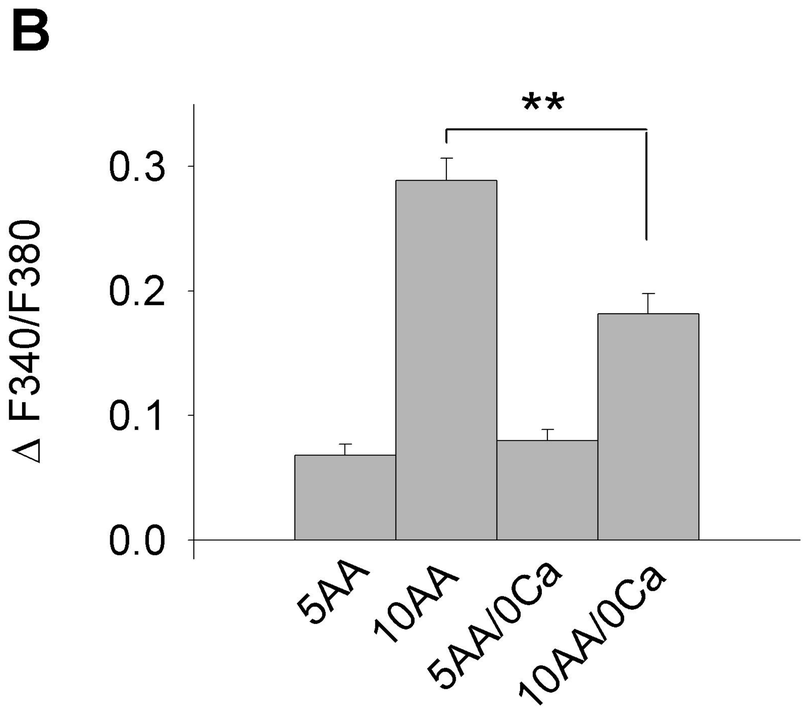
accumulation of fluorescent dye. C,D) A 5-min application of 30 μM BzATP alone did not cause any significant intracellular accumulation of fluorescent dye. E, F) Co-application of 30 μM BzATP *plus* 5 μM AA and 300 μM BzATP alone caused a significant intracellular accumulation of 6-FAM. Scale bar, 30 μm. Each experiment was repeated at least 7 times.

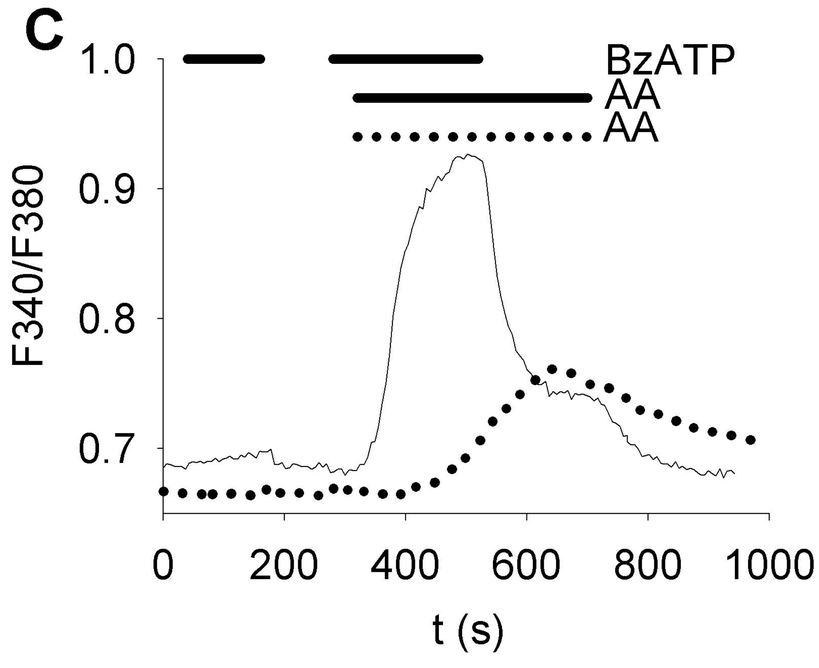
Fig. 5. Purinoceptor agonists and AA differently regulate $[Ca^{2+}]_i$ responses in HEK293 cells. A) Representative control HEK293 cell showing the transient $[Ca^{2+}]_i$ rise evoked by 10 μM ATP and no response to 10 μM BzATP (n=26). B) In the same cell, in the absence of extracellular Ca^{2+}, 10 μM AA induced a slowly activating transient $[Ca^{2+}]_i$ increase that was not modified upon re-addition of extracellular Ca^{2+}. C) In HEK293 cells transfected with P2X7-GFP, 1 μM BzATP did not elicit $[Ca^{2+}]_i$ increase, but subsequent application of 10 μM BzATP caused a robust $[Ca^{2+}]_i$ rise that rapidly returned to basal level upon BzATP removal (n=65). D) In the same cell of panel C, in the absence of extracellular Ca^{2+}, 10 μM AA induced a $[Ca^{2+}]_i$ response similar to that produced in non-transfected cells.

Fig. 6. Arachidonic acid induces potentiation of BzATP-evoked $[Ca^{2+}]_i$ increase in stably transfected HEK293 cells. A) HEK293 cells expressing rat P2X7 (rP2X7) were exposed to 1 μM BzATP alone and in conjunction with 5 μM AA (n=77). Dotted trace represents $[Ca^{2+}]_i$ response obtained from the same cell by subsequent application of 5 μM AA alone. B) Histogram illustrating the $[Ca^{2+}]_i$ increases evoked by individual applications of BzATP or AA, and 1 μM BzATP *plus* 5 μM AA in non-transfected (NT)

and stably transfected (T) HEK293 cells. Note the large difference in $[Ca^{2+}]_i$ elevation between P2X7-transfected HEK293 cells when stimulated with 1 μM BzATP or 1 μM BzATP *plus* 5 μM AA. Values represent means±SEM of at least 35 cells. Double asterisk denotes $p<0.01$. C) HEK293 cells expressing human P2X7 (hP2X7) were exposed to 3 μM BzATP in conjunction with 5 μM AA (n=58). D, *left*) Histogram illustrating the $[Ca^{2+}]_i$ increases evoked by individual applications of BzATP or AA, and 3 μM BzATP *plus* 5 μM AA in hP2X7- transfected HEK293 cells. Means±SEM of at least 43 cells. D, *right*) Histogram illustrating the current magnitude evoked by individual applications of BzATP (1, 10 μM), and BzATP *plus* 5 μM AA in rP2X7-transfected HEK293 cells. Means±SEM of at least 18 cells.







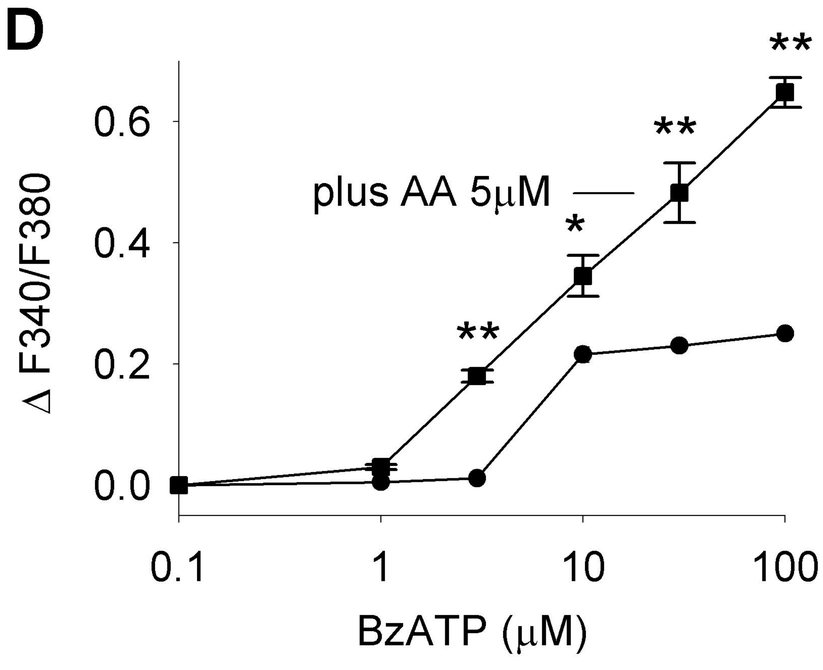


Fig.1

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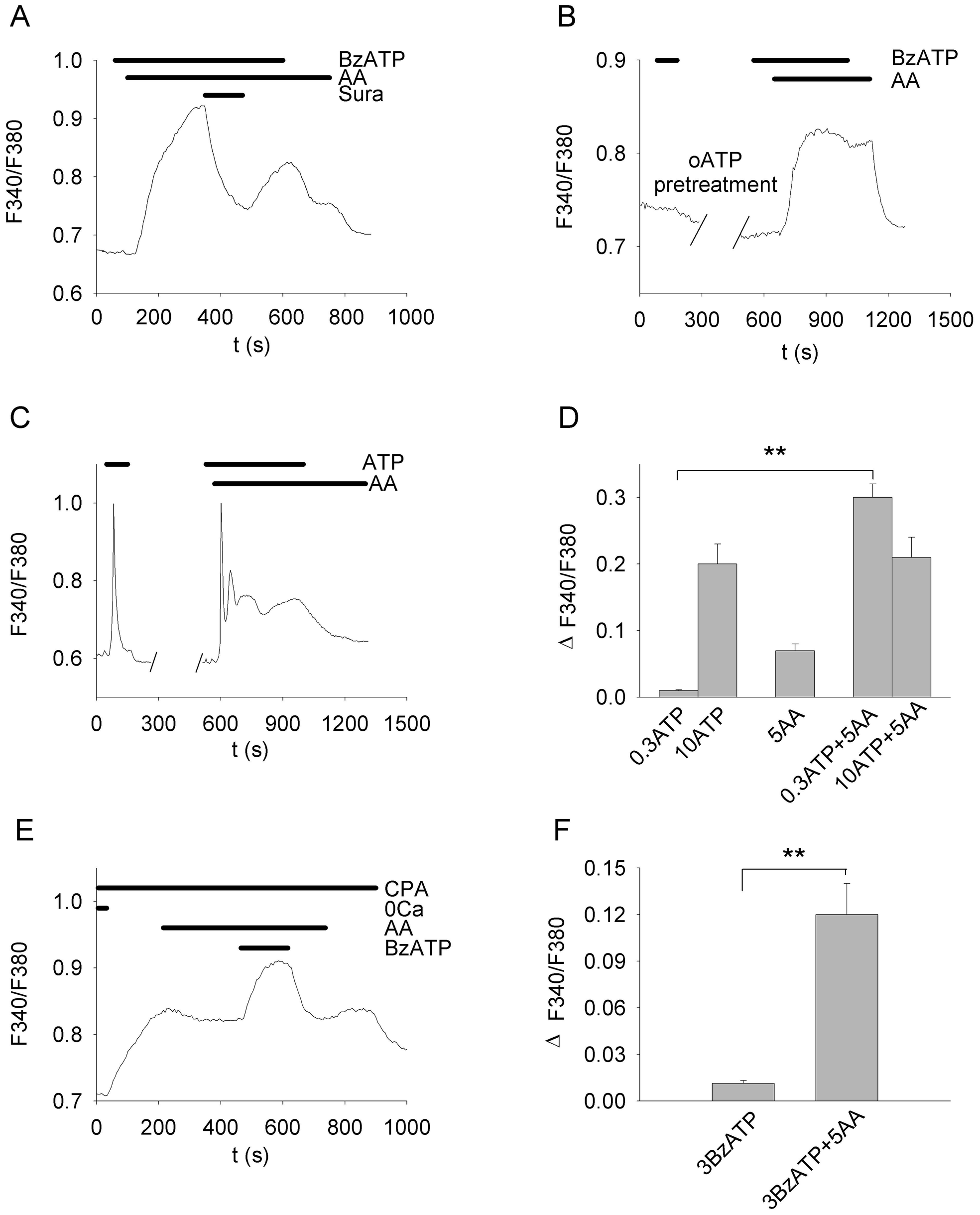

Fig.2

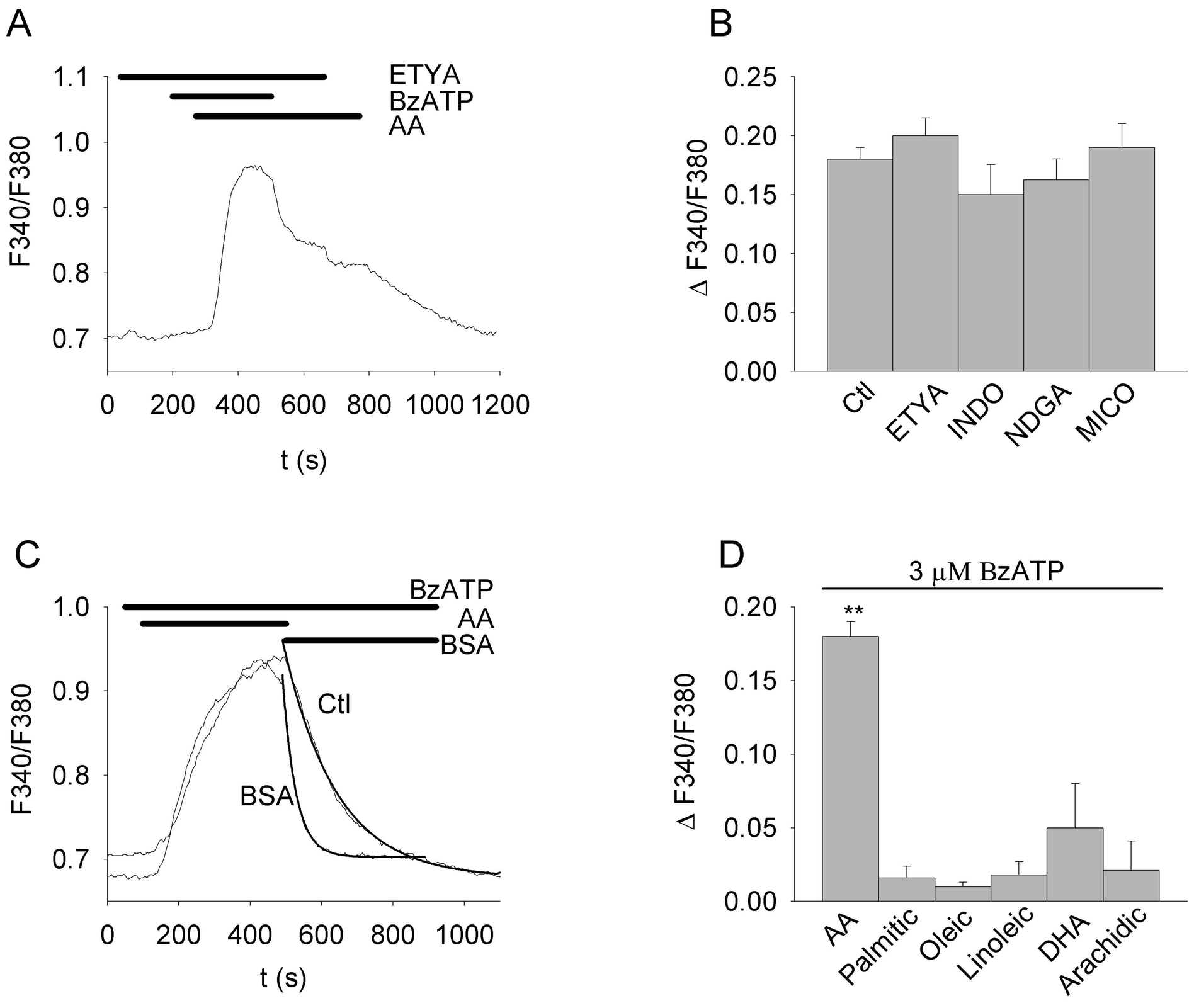

Fig.3

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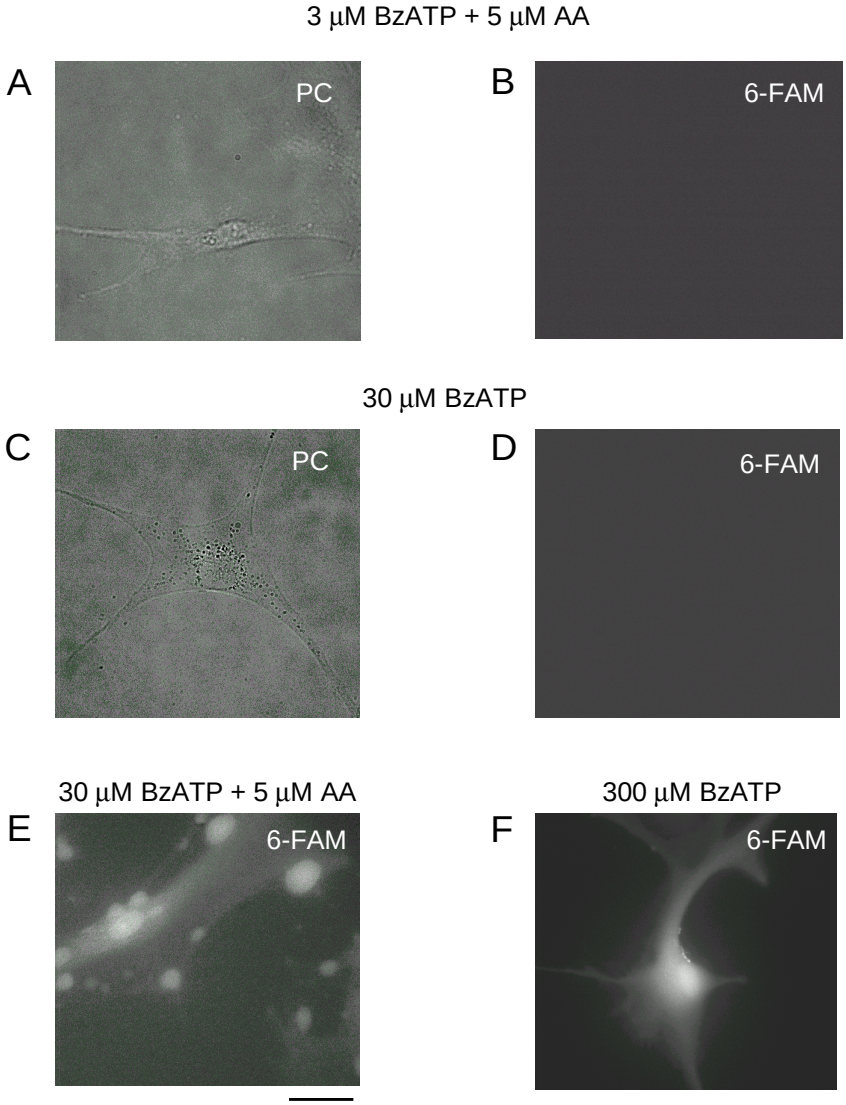

Fig.4

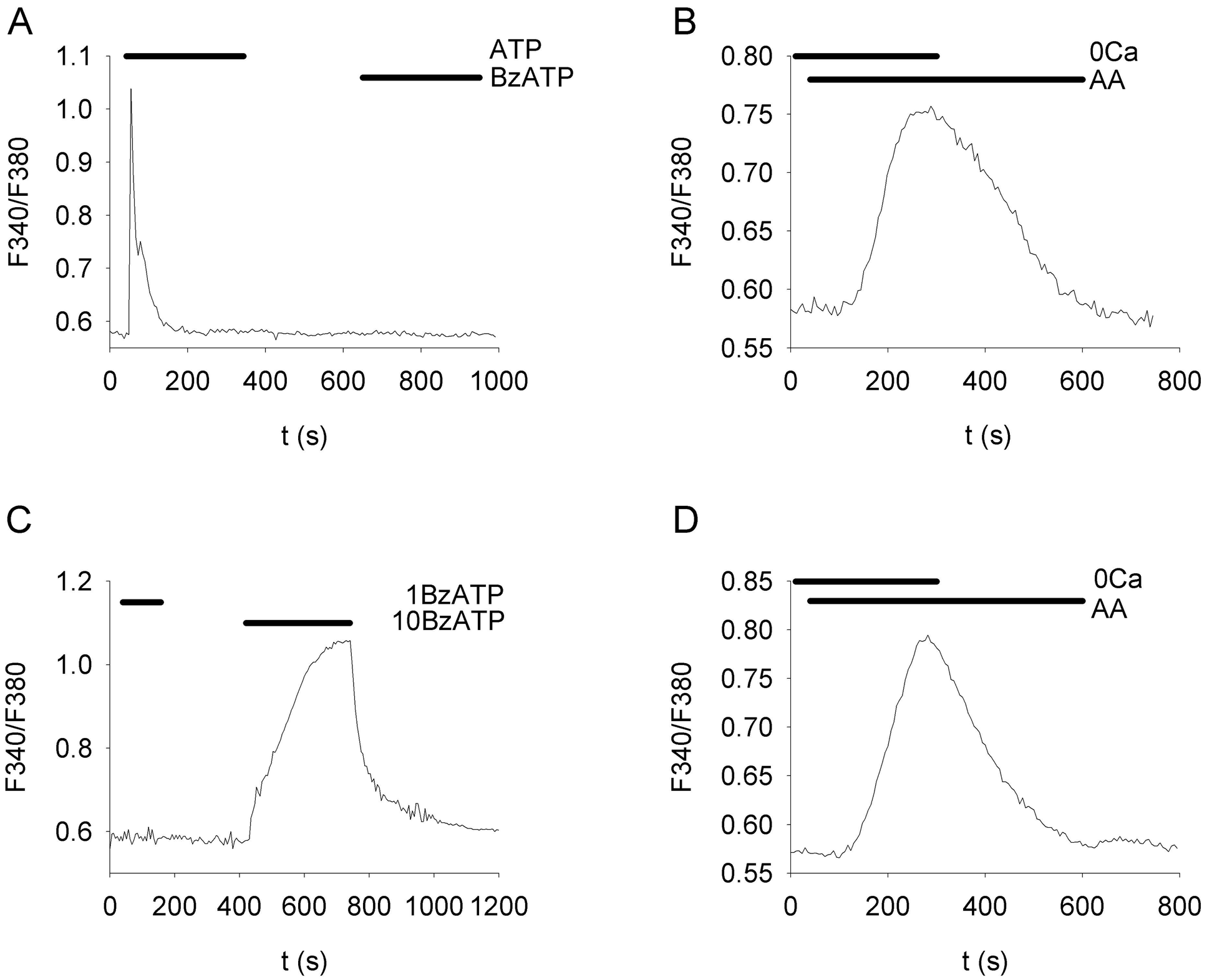

Fig.5

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Fig.6