ATP-induced, sustained calcium signalling in cultured rat cortical astrocytes: evidence for a non-capacitative, P2X7-like-mediated calcium entry

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Abstract **The receptor mechanisms regulating the ATP-induced free cytosolic Ca^{2+} concentration ($[Ca^{2+}]_i$) changes in cultured rat cortical type-1 astrocytes were analyzed using fura-2-based Ca^{2+} imaging microscopy. Upon prolonged ATP challenge (1–100 μM), astroglial cells displayed a biphasic $[Ca^{2+}]_i$ response consisting of an initial peak followed by a sustained elevation. Suramin and pyridoxalphosphate-6-azophenyl-2′,4′-disulfonic acid blocked both components, albeit to a different extent. By contrast, the selective P2X7 antagonist oxidized ATP irreversibly abrogated the sustained $[Ca^{2+}]_i$ signal without affecting the transient phase. Finally, astrocyte challenge with the selective P2X7 agonist 3′-*O*-(4-benzoyl)benzoyl-ATP evoked a sustained $[Ca^{2+}]_i$ elevation, which occluded that induced by ATP. We can conclude that in cultured cortical astrocytes the ATP-mediated sustained $[Ca^{2+}]_i$ rise does not implicate capacitative Ca^{2+} entry but involves Ca^{2+} influx through P2X7-like receptors.**

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Key words: Astroglia; P2 purinoceptor; Microfluorimetry; Calcium influx; P2X7 receptor

1. Introduction

Astrocytes are involved in multiple brain functions in physiological conditions, participating in neuronal development, synaptic activity and homeostatic control of the extracellular milieu [1]. Following pathological insults such as stroke, trauma and seizures as well as as a result of chronic neurological disorders (e.g. Alzheimer's disease and amyotrophic lateral sclerosis) astroglial cells undergo a vigorous activation, called reactive gliosis, which is characterized by increased cell proliferation and hypertrophy [2]. Several lines of evidence suggest that increased extracellular ATP, released from damaged or dying cells, may be implicated in promoting astrogliosis through stimulation of P2 purinoceptors [3,4]. Notably, ATP was shown to stimulate astrocyte proliferation upon activation of P2 receptors also in situ [5].

Activation of different subtypes of P2Y metabotropic receptors leads to the elevation of free cytosolic Ca^{2+} concentration ($[Ca^{2+}]_i$) mediated by phosphoinositide turnover-dependent Ca^{2+} release from intracellular organelles [6]. In different cell preparations this P2Y-mediated Ca^{2+} mobilization is accompanied by a substantial Ca^{2+} influx from the extracellular space via a pathway that is commonly referred to as capacitative Ca^{2+} entry (CCE), which is mediated by the opening of store-operated Ca^{2+} channels [7].

Although current models of receptor-activated Ca^{2+} entry focus on the capacitative mechanism, there is increasing evidence that this pathway is not the only one whereby Ca^{2+} can enter into the cells in response to activation of membrane receptors [8–10]. Recently, ionotropic P2 purinoceptors (P2X7) permeable to Ca^{2+} were functionally characterized in different cell types [6,11]. Hence, because of the complexity of the P2 purinoceptor-mediated $[Ca^{2+}]_i$ signalling, dissecting the relative contribution of the different $[Ca^{2+}]_i$-elevating pathways in each cellular context is of crucial importance in the attempt to determine the biological significance of the mechanisms regulating the intracellular Ca^{2+} homeostasis.

Astroglial cells express a variety of receptors for neurotransmitters and cell modulators, many of which are coupled to $[Ca^{2+}]_i$ rise via the phospholipase C/inositol triphosphate (PLC/IP_3) pathway [12,13]. Although CCE seems to be operational also in astroglia [14,15], the precise relationship between receptor activation, Ca^{2+} store depletion, and stimulation of Ca^{2+} entry has not been explored yet. Astrocytes both in vitro and in situ express PLC-coupled P2Y receptors [16]. Moreover, there is accumulating evidence that also astroglial cells possess P2X7 receptor, whose functional role, however, remains to be fully elucidated [17–20].

In the present study, we investigated the interplay between activation of ATP receptors, Ca^{2+} release from intracellular stores and Ca^{2+} entry across the plasma membrane in cultured rat cortical type-1 astrocytes. We were able to demonstrate that the sustained $[Ca^{2+}]_i$ increase observed following the transient $[Ca^{2+}]_i$ signal elicited upon stimulation of P2 purinoceptors with low micromolar concentrations of ATP is not mediated by CCE but is the result of extracellular Ca^{2+} influx through P2X7-like receptors.

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Abbreviations: $[Ca^{2+}]_i$, free cytosolic Ca^{2+} concentration; 2-MeSATP, 2-methylthio-ATP; β,γ-meATP, β,γ-methylene-ATP; Bz-ATP, 3′-*O*-(4-benzoyl)benzoyl-ATP; CPA, cyclopiazonic acid; Sura, suramin; PPADS, pyridoxalphosphate-6-azophenyl-2′,4′-disulfonic acid; oATP, oxidized ATP; CCE, capacitative Ca^{2+} entry; PLC, phospholipase C; IP_3, inositol triphosphate

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2. Materials and methods

2.1. Cell culture

Primary cultures of pure cortical rat astrocytes were prepared as previously described [21]. Briefly, cerebral cortices of 1–2-day-old pups devoid of meninges were triturated and placed in cell culture flasks containing DME-glutamax medium with 15% fetal calf serum and penicillin/streptomycin (100 U/ml and 100 μg/ml, respectively). Culture flasks were maintained in a humidified incubator with 5% CO_2 for 2–5 weeks. At confluence, astroglial cells were enzymatically dispersed (trypsin-EDTA) and replaced in 20-mm glass coverslips at a density of 5×10^3 per coverslip. All experiments were performed at room temperature (20–22°C) at day 3–8 after replating. Immunostaining for glial fibrillary acidic protein and the flat, polygonal shape of the cultured cells indicated that more that 95% were type-1 cortical astrocytes. For the preparation of the astrocytic cultures, all the materials used were from Gibco-BRL (Invitrogen, Italy). No evidence was found for variability in receptor properties over the period of cell culturing.

2.2. Solutions

During the experiments, the cells were continuously superfused using a multiple perfusion system allowing a flux of about 2.5 ml/min. The extracellular solution contained (mM): 135 NaCl, 5.4 KCl, 1.8 $CaCl_2$, 1 $MgCl_2$, 5 HEPES and 10 glucose (pH adjusted to 7.4 with NaOH). Ca^{2+}-free extracellular solution was prepared by replacing $CaCl_2$ with equimolar amounts of $MgCl_2$ and by adding 0.5 mM EGTA. The salt solutions and the chemicals for the fluorimetric determination of the $[Ca^{2+}]_i$ responses were obtained from Sigma-Aldrich Italy.

2.3. Imaging and data analysis

Intracellular calcium was monitored using the fluorescent Ca^{2+} indicator fura-2 AM. Cultures were loaded in extracellular solution (see above) supplemented with 10 μM fura-2 AM for 45 min at 37°C. Measurements of $[Ca^{2+}]_i$ of single cells were performed by using an inverted fluorescence microscope Nikon TE200 (Nikon, Tokyo, Japan) equipped with a dual excitation fluorometric imaging system (Hamamatsu, Sunayama-Cho, Japan). The excitation wavelength was alternated between 340 and 380 nm, and the emission fluorescence of selected astrocytes was passed through a 510-nm narrowband filter and recorded with a digital CCD camera Hamamatsu C4742-95-12ER. Monochromator settings, chopper frequency and complete data acquisition were controlled by Aquacosmos/Ratio software U7501-01 (Hamamatsu). The sampling rate was 0.25 or 0.5 Hz. At the end of each set of experiments, cells were permeabilized using the calcium ionophore 4-bromo-A23187 (20 μM) and the background fluorescence determined after exposure of the cells to an extracellular solution containing 2 mM Mn^{2+} instead of Ca^{2+} in order to quench the fura-2 fluorescence. The calibration of the 340/380-nm ratio in terms of free Ca^{2+} concentration was based on the procedure described by Grynkiewicz et al. [22].

2.4. 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 $P<0.05$.

3. Results

3.1. Effects of extracellular ATP on $[Ca^{2+}]_i$ in cultured cortical astrocytes

Calcium-imaging analysis showed that in cultured rat cortical type-1 astrocytes the mean resting $[Ca^{2+}]_i$ was 97.2 ± 7.4 nM (mean ± S.E.M.; $n=170$). Upon prolonged (up to 8 min) administration of 3 μM ATP, approximately 80% of the responding cells exhibited biphasic $[Ca^{2+}]_i$ responses, which consisted of an initial, large transient component, and a smaller sustained phase that showed little or no inactivation during ATP application (Fig. 1A). The amplitude of the initial $[Ca^{2+}]_i$ transient depended on ATP concentration with a half-maximal increase (EC_{50}) at 2.2 μM ATP and a Hill coefficient of 1.5 (Fig. 1B). The magnitude of the sustained phase was also dose-dependent with an EC_{50} of 0.9 μM and a Hill coefficient of 1.4 (Fig. 1C). The threshold for the Ca^{2+} elevation above basal levels was approximately 0.3 μM ATP for both the peak and the plateau phase and the maximal activation was reached in the presence of 100 μM ATP. In contrast to the transient $[Ca^{2+}]_i$ response, the sustained increase was rapidly abolished by omitting extracellular Ca^{2+} and addition of 0.5 mM EGTA, or application of either 10 μM La^{3+} or 100 μM Zn^{2+}, but was unaffected by 10 μM nifedipine, an antagonist of L-type voltage-activated Ca^{2+} channels. Thus, it can be concluded that whereas the $[Ca^{2+}]_i$ transient is due to ATP-evoked Ca^{2+} release from intracellular stores, the sustained $[Ca^{2+}]_i$ elevation is mediated by Ca^{2+} influx through the plasma membrane. Interestingly, the dynamics of the $[Ca^{2+}]_i$ responses triggered by ATP varied depending on the duration of agonist application as a brief challenge of about 20 s resulted only in a transient $[Ca^{2+}]_i$ rise followed by a fast decline to the resting level. Only when

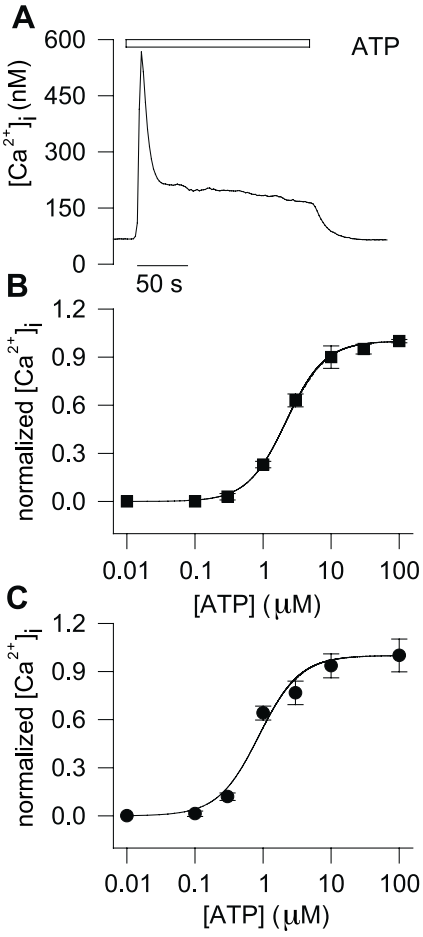

Fig. 1. ATP-mediated $[Ca^{2+}]_i$ responses in rat cortical astroglial cells. A: ATP (3 μM; horizontal bar) evoked $[Ca^{2+}]_i$ increase in cultured type-1 astrocytes. The typical response consisted of a transient increase followed by a sustained elevation in $[Ca^{2+}]_i$, which lasted on ATP application. B,C: Concentration dependence of the ATP-induced transient (B) and sustained $[Ca^{2+}]_i$ increases (C). The $[Ca^{2+}]_i$ elevations were normalized to the maximal responses, which were elicited by 100 μM ATP. The fit by the Hill equation yielded an EC_{50} of 2.2 μM (B) and 0.9 μM (C). Data are the average of several $[Ca^{2+}]_i$ values and each point is the mean of at least 12 astrocytes; error bars indicate S.E.M.

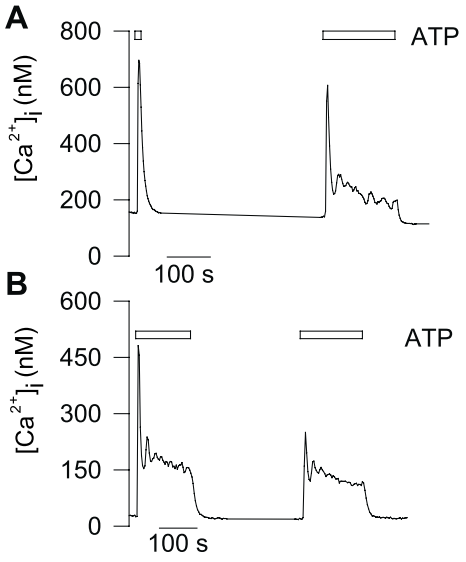

Fig. 2. The transient and sustained $[Ca^{2+}]_i$ rises are two distinct molecular events. A: Representative $[Ca^{2+}]_i$ responses obtained by changing the duration of the ATP (3 μM) application. Note that in the same astrocyte brief challenge (20 s) evoked only a transient response, whereas a long (3 min) application produced a biphasic $[Ca^{2+}]_i$ signal composed of a peak and a sustained phase. B: The $[Ca^{2+}]_i$ peak of the biphasic response was substantially diminished when a second ATP challenge was performed 5 min later. By contrast, the plateau was not significantly altered, to indicate that the two kinetically different $[Ca^{2+}]_i$ signals undergo differential desensitization patterns.

the application of ATP was prolonged, the $[Ca^{2+}]_i$ plateau could be observed ($n = 22$; Fig. 2A). The result that such differential behavior was observed in the same astrocyte rules out the possibility of an intercellular variability in the ATP-mediated $[Ca^{2+}]$ responses and supports the notion that different unrelated cellular mechanisms are involved. This indication was also corroborated by experiments showing that whereas the transient response was still largely desensitized after a 5-min time interval before a second ATP challenge, upon the same experimental paradigm the steady-state $[Ca^{2+}]_i$ elevation was unaffected compared to the first ATP application ($P > 0.05$, $n = 17$; Fig. 2B).

3.2. *Effects of P2 purinoceptor agonists on $[Ca^{2+}]_i$ in cortical astrocytes*

Since ATP is a non-selective P2 purinoceptor agonist, we next characterized the $[Ca^{2+}]$ responses elicited by agonists of P2Y and P2X receptor subfamilies. A prolonged bath application of the P2Y receptor agonist 2-methylthio-ATP (2-MeSATP; 10 μM; $n = 20$) evoked a transient $[Ca^{2+}]_i$ rise that decayed towards basal level with two time constants of 6.4 ± 0.8 and 130.1 ± 20.2 s ($n = 12$; Fig. 3A). The profile of these responses was not significantly different ($P > 0.05$) from that observed when the 1.8 mM extracellular Ca^{2+} was replaced and 0.5 mM EGTA added (time constants of 3.9 ± 0.1 and 106.4 ± 13.9 s; $n = 8$; data not shown). Noteworthy, this behavior of the $[Ca^{2+}]_i$ response was also observed upon astrocyte challenge with UTP (100 μM; $n = 22$; data not shown). Collectively, these results suggest that P2Y2/4 recep-

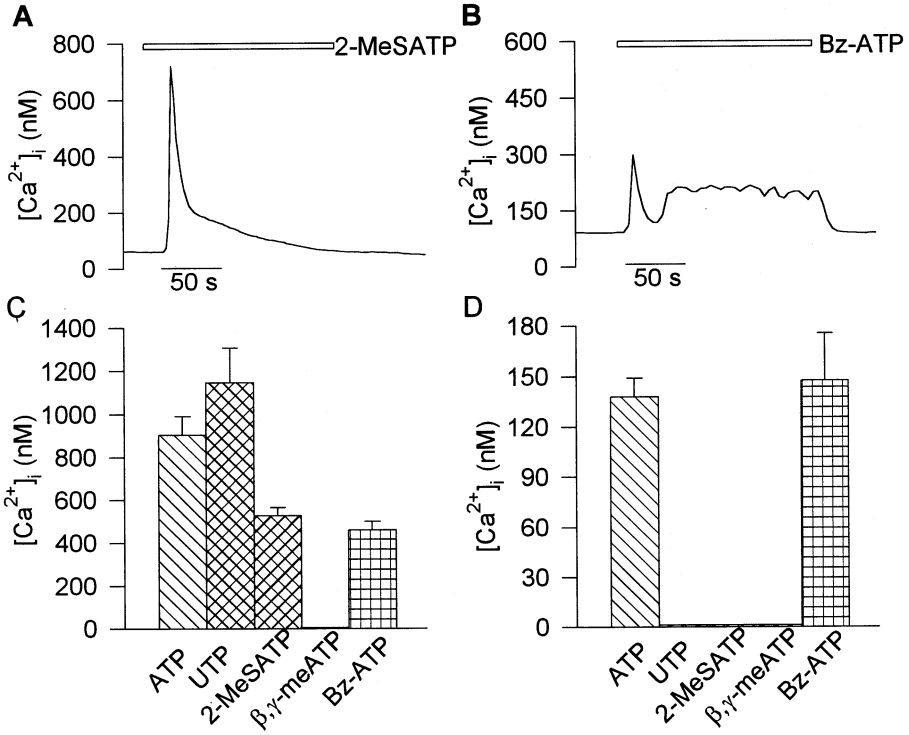

Fig. 3. Different P2 purinoceptor agonists evoke kinetically variable $[Ca^{2+}]_i$ responses. A: Bath application of the P2Y agonist 2-MeSATP (10 μM) evoked only a transient $[Ca^{2+}]_i$ increase that returned in about 3 min to the resting $[Ca^{2+}]_i$ level with double exponential temporal kinetics. B: The selective P2X7 agonist Bz-ATP (10 μM) evoked a small peak, which was followed by a $[Ca^{2+}]_i$ plateau similar to that induced by ATP application. C: Of the several P2 agonists tested the P2Y and P2X7 agonists generated a transient $[Ca^{2+}]_i$ response, whereas the P2X1/3 agonist βγ-meATP (10 μM) was ineffective. D: When the same agents were assayed for their ability to elicit a sustained $[Ca^{2+}]_i$ elevation, only ATP and Bz-ATP evoked a $[Ca^{2+}]_i$ plateau measured 4 min after starting the agonist application. Results are presented as $[Ca^{2+}]_i$ increases above basal levels and are expressed as means ± S.E.M.

tor subtypes are involved in the ATP-evoked transient $[Ca^{2+}]_i$ signal in cortical type-1 astrocytes. The observation that the $[Ca^{2+}]_i$ dynamics was not altered by omission of extracellular Ca^{2+} strongly indicates that under our experimental conditions activation of P2Y metabotropic receptors does not promote CCE. Whereas bath application of the P2X1/3 receptor agonist β,γ-methylene-ATP (β,γ-meATP; 10 μM; $n=13$) did not have any significant effect ($P>0.05$) on $[Ca^{2+}]_i$ homeostasis, the selective P2X7 agonist 3′-*O*-(4-benzoyl)benzoyl-ATP (Bz-ATP; 10 μM; $n=19$) evoked a small $[Ca^{2+}]_i$ peak followed by a plateau phase similar to that observed upon ATP application (Fig. 3B). Figs. 3C and D separately show the average transient and sustained $[Ca^{2+}]_i$ increases above basal levels, evoked by individual applications of UTP (100 μM), 2-MeSATP (10 μM), β,γ-meATP (10 μM), Bz-ATP (10 μM) compared with response elicited by 10 μM ATP. The data in Fig. 3D indicate that among the various P2 purinoceptor agonists tested only ATP and Bz-ATP evoked sustained $[Ca^{2+}]_i$ responses. The sustained $[Ca^{2+}]_i$ elevations above basal levels were 138.1 ± 11.0 nM for 10 μM ATP ($n=30$) and 147.5 ± 28.1 nM for 10 μM Bz-ATP ($n=19$).

3.3. Effects of Bz-ATP on $[Ca^{2+}]_i$ in cultured cortical astrocytes

When Bz-ATP was administered in the absence of extracellular Ca^{2+}, the cells exhibited only a transient $[Ca^{2+}]_i$ response and a sustained increase developed only after re-addition of extracellular Ca^{2+} (Fig. 4A; $n=12$). These data indicate that activation of the P2X7-like receptor in cortical astrocytes also promotes Ca^{2+} mobilization from intracellular stores. In the presence of 100 μM Zn^{2+} or 10 μM La^{3+} the Bz-ATP-induced $[Ca^{2+}]_i$ plateau was completely and reversibly abrogated (Fig. 4B; $n=14$). Moreover, removal of extracellular Mg^{2+} caused a 100% increase in the Bz-ATP-evoked $[Ca^{2+}]_i$ signal (data not shown; $n=8$). Next, we verified the additivity of the ATP- and Bz-ATP-mediated Ca^{2+} influx. As shown in Fig. 4C, Bz-ATP (10 μM) did not significantly ($P>0.05$) affect the ATP-induced $[Ca^{2+}]_i$ plateau ($n=13$), thus suggesting that the two agonists may share the same plasma membrane Ca^{2+} entry pathway. In order to discern whether the Bz-ATP-induced, sustained $[Ca^{2+}]_i$ response was mediated by store-dependent or store-independent pathways, we explored the effect of Bz-ATP on CCE induced by administration of cyclopiazonic acid (CPA), an inhibitor of the endoplasmic reticulum Ca^{2+}-ATPase. In Fig. 4D is shown that after depletion of the intracellular Ca^{2+} stores by CPA in Ca^{2+}-free solution (extracellular Ca^{2+} replaced and addition of 0.5 mM EGTA), a sustained $[Ca^{2+}]_i$ increase could be observed upon re-addition of Ca^{2+}. When Bz-ATP (10 μM) and CPA (10 μM) were co-applied in the presence of external Ca^{2+}, the evoked $[Ca^{2+}]_i$ rise was significantly enhanced by about 30% compared to CPA alone ($P<0.05$; $n=9$), which indicates that the Bz-ATP-evoked Ca^{2+}entry is not mediated by CCE.

3.4. Effects of P2 purinoceptor antagonists

To get further insights into the properties of the P2 purinoceptors involved in the ATP-induced $[Ca^{2+}]_i$ signals in cortical astrocytes, we analyzed the effects of suramin (Sura), pyridoxalphosphate-6-azophenyl-2′,4′-disulfonic acid (PPADS) and oxidized ATP (oATP) on both ATP- and Bz-ATP-stimulated $[Ca^{2+}]_i$ increases. As shown in Fig. 5A, 10 μM Sura, a broad-spectrum P2 antagonist, had differential effects on the transient and sustained Ca^{2+} signals: whereas Sura completely and reversibly blocked the transient $[Ca^{2+}]_i$ increase induced by 1 μM ATP, it reduced by about 70% the sustained component ($n=9$). The best fit of the dose–response curve relative to the Sura blockade of the ATP-induced $[Ca^{2+}]_i$ plateau depicts a half-effective concentration (IC_{50}) of 3.7 μM with a Hill coefficient of 1.2. Importantly, Sura (30 μM) completely depressed the plateau phase induced by 10 μM Bz-ATP (Fig. 5B; $n=12$). Likewise, PPADS (30 μM; $n=20$), another non-selective P2 antagonist, had inhibitory

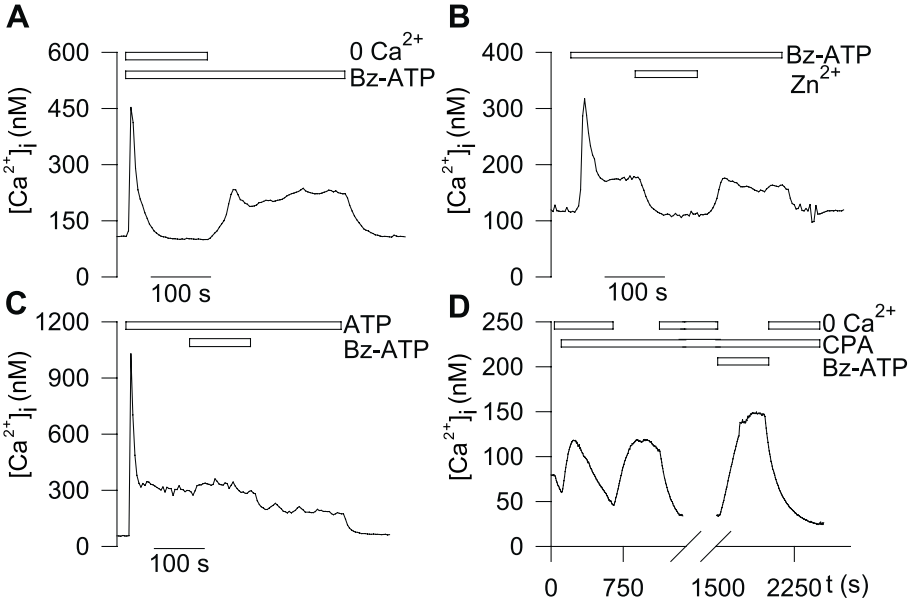

Fig. 4. Characterization of the Bz-ATP-evoked $[Ca^{2+}]_i$ signal. A: When Bz-ATP (10 μM) was administered in the absence of extracellular Ca^{2+} only a transient $[Ca^{2+}]_i$ elevation was observed; upon re-addition of extracellular Ca^{2+}, $[Ca^{2+}]_i$ rose to a steady-state level, which lasted over the Bz-ATP challenge. B: The plateau phase of the Bz-ATP-induced $[Ca^{2+}]_i$ increase was reversibly inhibited by application of 100 μM Zn^{2+}. C: The stimulation of the sustained $[Ca^{2+}]_i$ signal by Bz-ATP was not additive to that of ATP (10 μM). D: After exposure to CPA in a Ca^{2+}-free solution to evoke CCE, the $[Ca^{2+}]_i$ responses elicited following Ca^{2+} re-addition were greater upon co-application of Bz-ATP.

effects on the ATP- and Bz-ATP-induced sustained $[Ca^{2+}]_i$ elevations which were qualitatively similar to those obtained with Sura (data not shown). The likely involvement of P2X7 receptors as molecular entity underlying the sustained $[Ca^{2+}]_i$ increase was further corroborated by the observation that the selective P2X7 antagonist oATP irreversibly reduced the ATP-elicited $[Ca^{2+}]_i$ plateau without affecting the transient component. Fig. 5C shows that after a 2-h preincubation with 100 μM oATP, ATP (10 μM) induced only a transient $[Ca^{2+}]_i$ elevation ($n = 11$). The blockage was irreversible as even upon prolonged oATP washing, a new ATP challenge evoked solely a transient response. It is worth noting that Sura, PPADS and oATP did not have any effect on the $[Ca^{2+}]_i$ signal elicited by CPA-mediated CCE ($n = 15$; data not shown). Taken together, these results add support to the tenet that in cultured astroglial cells the ATP-induced transient $[Ca^{2+}]_i$ elevation and the $[Ca^{2+}]_i$ plateau are dependent on the activation of different subtypes of P2 purinoceptors, and suggest that the sustained $[Ca^{2+}]_i$ signalling is mediated by activation of Ca^{2+}permeable P2X7-like receptors.

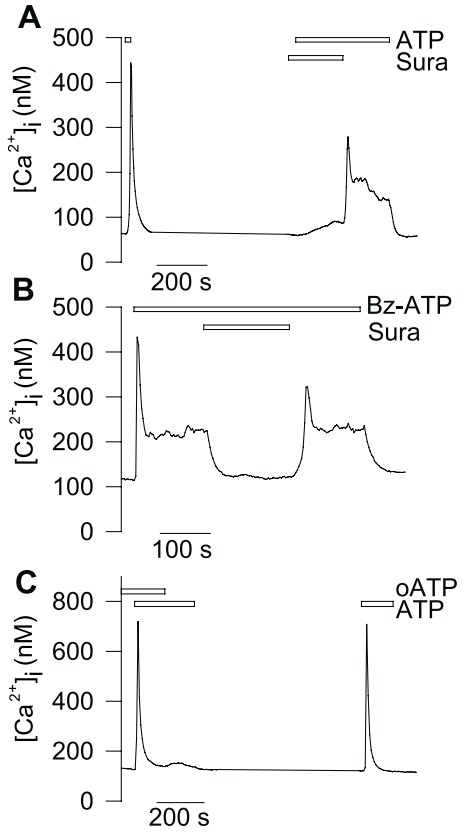

Fig. 5. Effects of P2 purinoceptor antagonists on astroglial $[Ca^{2+}]_i$ increase. A: ATP-induced $[Ca^{2+}]_i$ responses in the absence or presence of 10 μM Sura. Sura was applied 30 s before the addition of 1 μM ATP. At this concentration Sura had differential effects on the transient and sustained $[Ca^{2+}]_i$ elevations, being more effective on the peak. B: A higher Sura concentration (30 μM) fully blocked the $[Ca^{2+}]_i$ plateau induced by 10 μM Bz-ATP. This concentration also completely abrogated the ATP-mediated sustained $[Ca^{2+}]_i$ elevation. C: The $[Ca^{2+}]_i$ signal evoked by 10 μM ATP after a 2-h astrocyte preincubation with 100 μM oATP consisted only of a transient response. Note the irreversibility of the oATP effect as depicted by the observation that a second ATP challenge after more than 10 min of oATP washout evoked only a $[Ca^{2+}]_i$ transient.

4. Discussion

In the present study we provide evidence that in cultured rat type-1 cortical astrocytes the sustained $[Ca^{2+}]_i$ elevation which follows the $[Ca^{2+}]_i$ transient induced by prolonged application of ATP at concentrations lower than 100 μM is mediated by Ca^{2+} influx across the plasma membrane caused by activation of P2X7 purinoceptors. We also show that CCE contributes marginally, if at all, to the ATP-induced sustained $[Ca^{2+}]_i$ rise. In some experiments $[Ca^{2+}]_i$ oscillations were superimposed on the plateau $[Ca^{2+}]_i$ responses. There is evidence that in astrocytes metabotropic receptor-mediated $[Ca^{2+}]_i$ oscillations depend on agonist concentration and that extracellular Ca^{2+} is required for such oscillatory activity (for a review, see [13]). In our experiments the appearance of oscillatory responses did not seem to depend on ATP concentration. In this context, further work will be necessary to identify the cellular mechanisms underlying $[Ca^{2+}]_i$ oscillations and to address their biological significance.

Three main categories of Ca^{2+}-permeable channels have been reported to mediate Ca^{2+} entry from the extracellular space [8]: voltage-operated Ca^{2+} channels, ligand-gated non-specific cation channels and receptor-dependent Ca^{2+} channels further subdivided into store-operated (or capacitative Ca^{2+} channels) and non-capacitative Ca^{2+} channels regulated independently of store depletion. The existence of store-operated channels in astrocytes and glioma cells has been recently demonstrated [10,14,15,23,24]. Wu et al. [24] reported that intracellular ATP levels are important for the maintenance of the $[Ca^{2+}]_i$ plateau, and Rzigalinski et al. [15] suggested that 5,6-epoxyeicosatrienoic acid is a molecular component of the intracellular signalling cascade coupling store depletion to Ca^{2+} entry in cultured cortical astrocytes. Moreover, the involvement of non-capacitative Ca^{2+} entry mechanisms was suggested to contribute to the hypoosmolarity- and histamine-induced $[Ca^{2+}]_i$ responses in cultured rat astrocytes [10,23]. Finally, a novel mechanism of $[Ca^{2+}]_i$ elevation mediated by Ca^{2+} influx has been demonstrated to underlie part of the astrocytic $[Ca^{2+}]_i$ signal induced by activation of P2 purinoceptors. Such a mechanism requires the presence of a plasmalemma Ca^{2+}-permeable ionotropic receptor (P2X7), which is activated by high ATP concentrations and is also able to generate several biochemical responses [17–20]. The presence of CCE through store-operated Ca^{2+} channels in our astrocytic cultures could be depicted from experiments in extracellular Ca^{2+}-free conditions by using CPA to empty Ca^{2+} stores. Under these conditions, upon Ca^{2+} re-addition astrocytes displayed a sustained Ca^{2+} influx, which was not affected by the ATP receptor antagonists. The data that the ATP-induced $[Ca^{2+}]_i$ plateau was abolished by divalent cations cannot be taken as an indication for the involvement of CCE in the ATP-elicited sustained $[Ca^{2+}]_i$ rise because also recombinant P2X7 channels were shown to be inhibited by micromolar concentrations of various divalent cations [25]. An argument suggesting the involvement of P2X7 receptors rather than CCE in the ATP-induced sustained $[Ca^{2+}]_i$ signal is the result that, in addition to the blockade by the broad-spectrum ATP receptor antagonists Sura and PPADS, the $[Ca^{2+}]_i$ plateau was nearly completely inhibited by application of the selective P2X7 antagonist oATP. Finally, the selective P2X7 agonist Bz-ATP induced a Ca^{2+} entry that occluded the Ca^{2+} influx elicited by ATP. Hence, even though CCE can be

activated under our experimental conditions, it does not contribute substantially to the ATP-mediated sustained $[Ca^{2+}]_i$ response. This finding is in contrast with previous studies which, based on divalent sensitivity, postulated that the ATP-induced $[Ca^{2+}]_i$ plateau was mediated by a store-operated Ca^{2+} influx [26]. A notable difference between our data and those previously reported in other astrocytic preparations, in which higher concentrations were utilized (>100 μM), is the requirement of relatively low levels of ATP (<100 μM) to cause a Ca^{2+} influx mediated by P2X7-like receptors [18,20]. The reason for this discrepancy is unknown. However, since in those studies a type-2 astrocytic cell line was used, the differential expression of P2X7 isoforms with different ATP sensitivity cannot be ruled out. By contrast, the possibility that heteromeric channels formed by the subunit interactions between P2X7 and other P2X receptor subtypes present in cultured cortical astrocytes is unlikely because recent work had shown that in astroglial cells P2X7 receptor exists as a monomer [27].

It is well established that in vitro extracellular ATP stimulates proliferation and causes changes in astrocyte morphology, including process extension and thickening [28–30]. In vivo, such a change in astrocytic morphology is called reactive astrogliosis and is observed as a result of several pathological insults [31,32]. The hypothesis that P2 purinoceptors may play a role in the astrocyte reaction to brain injuries was recently confirmed by the observation that in vivo activation of P2 receptors causes astrocyte proliferation and hypertrophy [5]. The activation of a G protein-coupled P2Y-like receptor was proposed to underlie the proliferative action of ATP [33]. Moreover, by using various receptor antagonists, the possible role of P2X7 in this trophic effect could be ruled out [34]. Finally, a likely activation of P2Y receptor was shown to have a causative role in astrocytic proliferation in vivo [5]. Our data that already low micromolar concentrations of ATP evoke a significant, sustained elevation of cytoplasmic calcium through extracellular Ca^{2+} entry via P2X7 receptors suggest that this ionotropic purinoceptor may also have a role in the physiological regulation of astroglial syncytium. Notably, there is evidence that P2X7 receptor stimulation activates various intracellular signalling pathways [18,19]. Moreover, stimulation of P2X7 receptor in cultured astrocytes was reported to increase the expression of chemokine MCP-1 [19] and to regulate the release of purines [17]. Since the regulated, Ca^{2+}-dependent secretion of neurotransmitters and neuromodulators from astroglia has been postulated to play a crucial role in neuron–astroglia communication [1], and because of the importance that ATP-mediated $[Ca^{2+}]_i$ dynamics may have in such interaction [35], this study indicates that astroglial P2X7 receptor may be involved in the control of the excitability of the neuronal network.

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