Purinergic Receptors Mediate Two Distinct Glutamate Release Pathways in Hippocampal Astrocytes*

The purinergic P2X<sub>7</sub> receptor (P2X<sub>7</sub>-R) can mediate glutamate release from cultured astrocytes. Using patch clamp recordings, we investigated whether P2X<sub>7</sub>-Rs have the same action in hippocampal astrocytes in situ. We found that 2- and 3-O-(4-benzoylbenzoyl)ATP (BzATP), a potent, although unselective P2X-R agonist, triggers two different glutamate-mediated responses in CA1 pyramidal neurons; they are transient inward currents, which have the kinetic and pharmacological properties of previously described slow inward currents (SICs) due to Ca<sup>2+</sup>-dependent glutamate release from astrocytes, and a sustained tonic current. Although SICs were unaffected by P2X<sub>7</sub>-R antagonists, the tonic current was inhibited, was amplified in low extracellular Ca<sup>2+</sup>, and was insensitive to glutamate transporter and hemichannel inhibitors. BzATP triggered in astrocytes a large depolarization that was inhibited by P2X<sub>7</sub>-R antagonists and amplified in low Ca<sup>2+</sup>. In low Ca<sup>2+</sup> BzATP also induced lucifer yellow uptake into a subpopulation of astrocytes and CA3 neurons. Our results demonstrate that purinergic receptors other than the P2X<sub>7</sub>-R mediate glutamate release that evokes SICs, whereas activation of a receptor that has features similar to the P2X<sub>7</sub>-R, mediates a sustained glutamate efflux that generates a tonic current in CA1 neurons. This sustained glutamate efflux, which is potentiated under non-physiological conditions, may have important pathological actions in the brain.

Glutamate is the principal mediator of excitatory neurotransmission in the central nervous system as well as a recognized excitotoxic agent that can lead neurons and astrocytes to death when present at excessive extracellular concentrations. The ability to release this transmitter is diffusion-limited at physiological glutamate concentrations as well as in the excitotoxic action of glutamate. Although this latter issue remains to be proved, increasing evidence indicates that astrocyte-derived glutamate has complex actions on neurons exerting a modulatory role on synaptic transmission. For example, in the retina the release of glutamate from astrocytes modulates ganglion cell spike activity driven by light stimulation, most likely through a presynaptic action. In the hippocampus, it modulates excitability of interneurons and potentiates inhibitory transmission. On the other hand, at physiological concentrations of extracellular Ca<sup>2+</sup>, ATP is known to trigger a significant release of glutamate from astrocytes (19, 20), which in this case depends on a Ca<sup>2+</sup>-dependent pathway. Indeed, by activating both ionotropic, Ca<sup>2+</sup>-permeable P2XRs, including the P2X<sub>7</sub>-R and/or metabotropic P2YRs, ATP can evoke an intracellular Ca<sup>2+</sup> increase that in turn mediates glutamate release.

The aim of this study was to gain insights into the role of the purinergic receptor P2X<sub>7</sub> in the generation of glutamate release from in situ astrocytes. Results obtained in patch clamp recordings from neurons and astrocytes of acute hippocampal slices allow us to conclude that P2X<sub>7</sub>-R activation is not involved in the episodic glutamate release that triggers SICs. In contrast, a P2X<sub>7</sub>-like receptor, possibly expressed in astrocytes and neurons, contributes significantly to increasing the extracellular concentration of glutamate, in particular when extracellular Ca<sup>2+</sup> decreases to very low levels.

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The abbreviations used are: NMDAR, N-methyl-D-aspartate (NMDA) receptor; P2X<sub>7</sub>-R, purinergic P2X<sub>7</sub> receptor; Ca<sup>2+</sup>, intracellular Ca<sup>2+</sup> concentration; BzATP, 2- and 3-O-(4-benzoylbenzoyl)ATP; SIC, slow inward current; TTX, tetrodotoxin; LY, lucifer yellow; D-AP5, D-(-)-2-amino-5-phosphonopentanoic acid; NBQX, 2,3-dioxo-6-nitro-1,2,3,4-tetrahydrobenzof[1,4]oxazine-5-sulfonamide; TBOA, DL-threo-β-benzylaspartate; αβ-mATP, αβ-methylene ATP; BBG, Brilliant Blue G; OXATP, adenosine 5′-triphosphate-2,3-dialde- hyde; AMPA, α-amino-3-hydroxy-5-methyl-4-isoxazolpropionic acid.
MATERIALS AND METHODS

Slice Preparation—Transverse hippocampal slices (350–400 μm) were prepared as previously described (4, 21) from Wistar rats at postnatal days 10–23. After cutting, slices were incubated at 37 °C for a recovery period of at least 1 h. Slice cutting and incubation was performed with the following physiological saline solution: 120 mM NaCl, 3.2 mM KCl, 1 mM KH₂PO₄, 26 mM NaHCO₃, 2 mM MgCl₂, 1 mM CaCl₂, 2.8 mM glucose, 2 mM sodium pyruvate, and 0.6 mM ascorbic acid at pH 7.4 with 95% O₂, 5% CO₂. Slices incubation with 300 μM OxATP for 2–3 h and with 2–4 μM BBG for either 30 min or 1 h was carried out at 37 °C. When incubated with BBG for 30 min, slices were preincubated for 30 min in saline at 37 °C. As appropriate controls in OxATP and BBG experiments, we used slices maintained at 37 °C for either 1 or 2–3 h, respectively, and the recordings from these slices was performed the same days of the recordings from BBG- or OxATP-incubated slices. Recordings were also performed from additional neurons from control slices on days different from the days in which BBG and OxATP experiments were performed. Because the mean values of SIC frequency and tonic current amplitude from the different control experiments were similar, data from these experiments were pooled together.

Patch Clamp Recordings and Data Analysis—Most of the experimental procedures are similar to that described previously (11). In brief, during recordings slices were perfused with the following saline solution: 120 mM NaCl, 3.2 mM KCl, 1 mM KH₂PO₄, 26 mM NaHCO₃, 2 mM CaCl₂, 2.8 mM glucose, and 1 mM glycine at pH 7.4 with 95% O₂, 5% CO₂. Low Ca²⁺ solution was obtained by replacing CaCl₂ with EGTA (0.25 mM). Intracellular pipette solution was 145 mM potassium gluconate, 2 mM MgCl₂, 5 mM EGTA, 2 mM Na₂ATP, 0.2 mM NaGTP, and 10 mM HEPES to pH 7.4 with KOH. Slices were viewed with an upright Zeiss Axioscope microscope (Carl Zeiss Spa, Milan, IT) equipped with differential interference Nomarski optics, a CCD camera (COHU Inc., San Diego, CA), and a mercury light source for fluorescence excitation. Data were amplified and filtered at 1 kHz with one or two Axopatch-200B amplifiers and sampled at 5 kHz with a Digidata 1200 interface (Axon instruments, Union City, CA). The liquid junction potential at the pipette tip was −15 mV. This value should be added to all voltages to obtain the correct value of the membrane potential in whole-cell configuration. Series resistance (6 < Rₛ < 15 megaohms) was monitored throughout each experiment, and no compensation of Rₛ was applied. All experiments were performed at either room temperature or 35 °C and in the presence of TTX. Astrocytes were identified on the basis of both morphological and electrophysiological criteria. Astrocytes typically have small and round cell soma (diameter 6–10 μm), lack optically apparent large processes, do not fire action potentials upon application of depolarizing current pulses, and have a low input resistance (mean: 15.5 ± 1.9 megaohms, n = 24) and highly negative resting potentials (mean, −77.3 ± 0.7 mV, n = 24). All the astrocytes considered in this study exhibited a linear I–V relationship, typical of passive astrocytes (22). Neurons were voltage-clamped at −60 mV and astrocytes at −75 mV. Clampfit 8.2 (Axon instrument) and Origin 6.0 (Microwal Software, Northampton, MA) software were used for data analysis and fitting.

Transient inward currents with rise time slower than 10 ms and amplitude greater than −20 pA were classified as SICs. SIC frequency, amplitude, and kinetics were measured as described previously (11). In pair recording experiments, the time interval between two SICs was measured as the time interval between the onset of the current in cell 1 and the onset of the current in cell 2. Background noise amplitude was calculated as the peak-to-peak amplitude. Student’s t test was performed to determine statistical significance. Data are expressed as mean ± S.E.

RESULTS

P2X₇ Receptor-mediated Glutamate Release

Ca²⁺ Imaging and Dye Uptake—Slice incubation with 10 μM Rhod-2 AM (Molecular Probes, Eugene, OR) and 0.02% pluronic for 50 min at 37 °C under mild stirring resulted in the selective loading of the Ca²⁺ indicator into astrocytes (23). A confocal laser scanning microscope (TCS SP2 RS, Leica, Mannheim, Germany) was used for monitoring the BzATP-induced [Ca²⁺], change in these cells. Slices were continuously perfused at room temperature with the same extracellular solution as used in electrophysiological recording with 0.2 mM sulfipyrazone. The sampling rate was 2–4 s, and 8 images were averaged for each frame. Rhod-2 fluorescence was excited at 543 nm, and emitted light above 550 nm was collected. The fluorescent signal at a given time point was expressed as ΔF/F = (F₁ − F₀)/F₀, where F₁ and F₀ are the values of the fluorescence in astrocytes at rest and, respectively. No background subtraction was applied.

Dye uptake experiments were carried out incubating slices for 5–15 min with 1 μM TTX, 0.5 mg/ml lucifer yellow (LY) with or without 100 μM BzATP in the same solution used in patch clamp recording experiments under mild stirring and continuous bubbling with 95% O₂, 5% CO₂ to maintain pH 7.4. Incubation was terminated by washing in physiological saline, and fluorescence imaging was performed within 30 min. Fluorescence images were obtained with the same Leica confocal microscope using 458-nm excitation for LY. Cells were visually identified from the differential interference contrast image, and fluorescence intensity was measured from individual cells as the average intensity of fluorescence in a region of interest corresponding to the cell soma. No background subtraction was applied. In the 8-bit scale, a value of fluorescence intensity corresponding to 50 arbitrary units (3-fold the average background noise value: 17.7 ± 0.8 arbitrary units, n = 475) was chosen as the threshold for classifying positive cells. Background noise was not different in control slices and in slices incubated with BzATP or BzATP and low Ca²⁺. Because of possible damage caused by slice cutting procedures, cells located close to the surface may unspecifically take up the dye. Therefore, cells positively loaded with the dye were considered only when they were located below the first 10–15 μm from the surface.

Drugs—D-AP5, NBQX, 2-methyl-6-((phenylethynyl)pyridine hydrochloride, LY367385 ((S)-(+) - α-aminoo-4-carboxy-2-methyl-benzeneacetic acid), TBOA, and TTX were purchased from Tocris Cookson (Buckhurst Hill, UK), and BzATP, α,β-mATP, BBG, OxATP, LY, and carboxenolone were from Sigma. All chemicals were dissolved in water or Me₂SO and then diluted in the recording physiological solution just before use.
Both the transient currents (Fig. 2A) and the tonic current associated with noise increase (Fig. 2, B and C) evoked by BzATP were reversibly blocked by the NMDAR antagonist D-AP5 (50–100 μM) and can, thus, be attributed to the activation of NMDARs. These results clearly indicate that slice perfusion with BzATP triggers the release of glutamate that evokes in CA1 pyramidal neurons both transient and sustained activation of the NMDARs.

BzATP-induced noise increase is not due to an increase in the spontaneous synaptic release of glutamate since in the presence of D-AP5 and TTX, the frequency and amplitude of AMPA-mediated miniature excitatory postsynaptic currents evoked in the same neurons by Schaffer collateral stimulation (rise time, 6.7 ± 0.5 ms, n = 22) were not significantly changed (12 ± 9 versus 15 ± 13 events/min, n = 4; −7.7 ± 0.2 pA, n = 115, versus −8.1 ± 0.3 pA, n = 176; p > 0.2).

Glutamate Release from Astrocytes Mediates the Transient Neuronal Response—Elevations in the [Ca^{2+}]_i, evoked in hippocampal CA1 astrocytes by various stimuli, including purinergic receptor agonists, have been recently shown to trigger a pulsatile release of glutamate from these cells (11). Glutamate released from activated astrocytes acts primarily on extra-synaptic NMDARs of CA1 pyramidal neurons to trigger episodic inward currents with characteristic slow kinetics that we have called SICs (11). The transient, slow events evoked by BzATP in CA1 pyramidal neurons described above are mediated exclusively by NMDARs and are insensitive to 1 μM TTX. Therefore, they have the same pharmacological profile of SICs (11) as well as of the slow currents described by Angulo et al. (10). As shown in Fig. 3A, the mean rise time of BzATP-induced transient currents was 70.1 ± 7.9 ms (n = 54), one order of magnitude larger than that of excitatory postsynaptic currents evoked in the same neurons by Schaffer collateral stimulation (rise time, 6.7 ± 0.5 ms, n = 22). The mean decay time was 376.5 ± 24.3 ms (Fig. 3B; n = 54), which is slower than that of the excitatory postsynaptic currents from the same neurons (τ_r = 17.9 ± 6.7 ms, n = 54, versus 195.5 ± 14.7 ms, n = 22). The amplitude of these events can reach several hundred pA, with a mean of −115.9 ± 15.6 pA (Fig. 3C; n = 54). These features are also typical of SICs, providing further evidence for the classification of BzATP-evoked transient currents as SICs.

An additional feature of SICs is that they occur with a high degree of synchrony in different pyramidal neurons (10, 11). We investigated this issue by using patch clamp pair recordings from two pyramidal neurons. In two of the four pairs that displayed the transient currents upon BzATP stimulation, synchronous events were observed (Fig. 3D). The inter-event time interval histogram (Fig. 3E) shows that, similar to SICs, 42% of these events (40 of 96) are synchronized in a time window of 200 ms. Taken together, these data suggest that the BzATP-induced transient currents have the same pharmacological and biophysical properties of SICs, i.e. the...
NMDAR-mediated, slow inward currents that are triggered in CA1 pyramidal neurons by glutamate released from activated astrocytes.

SIC and Tonic Current Generation Is Dependent on the Activation of Different Purinergic Receptors—Given that BzATP is known to be a strong agonist of the P2X₇R, we next investigated the possible involvement of this receptor in the generation of the BzATP-induced SICs and tonic current. To this aim, we used OxATP (300 μM, top) and BBG (2 μM, bottom), respectively. 8, average frequency of BzATP-evoked SICs in control condition (n = 13) and in slices incubated in OxATP (n = 8) or BBG (n = 7). C, average amplitude values of the BzATP-evoked tonic current in control slices (n = 13) and in slices incubated in OxATP (n = 15) or BBG (n = 13). *, p < 0.05; **, p < 0.01. D, mean amplitude value of the background noise during BzATP application and after its washout in slices incubated with OxATP (left; n = 15) or BBG (right; n = 13). Data are normalized to the noise amplitude before BzATP application.

Support for this hypothesis derives from the observation that other stimuli used to trigger SICs, such as (5)-3,5-dihydroxyphenylglycine, prostaglandin E₂, or photolysis of caged-Ca²⁺, did not evoke a tonic current associated with an increase in background noise (11). Interestingly, 100 μM α,β-mATP, a purinergic agonist different from BzATP significantly different from controls (Fig. 4B; Table 1). Interestingly, under these conditions and in all the neurons tested, BzATP failed to trigger a tonic current (Fig. 4C) or an increase in background noise (Fig. 4D). These data demonstrate that, on the one hand, SICs generation is not mediated by P2X₇ receptors and, on the other, that the generation of the tonic current is dependent upon the activation of a purinergic receptor type that is blocked by OxATP and BBG.
P2X7 Receptor-mediated Glutamate Release

TABLE 1  
Amplitude and kinetics of SICs are independent of P2X7R activation

| Condition       | Amplitude (pA) | Rise time (ms) | Decay time (ms) | n  |
|-----------------|----------------|----------------|-----------------|----|
| BzATP           | −115.9 ± 15.6  | 70.1 ± 7.9     | 376.5 ± 24.4    | 54 |
| BzATP + BBG     | −94.9 ± 9.8    | 53.3 ± 8.0     | 347.5 ± 48.4    | 47 |
| BzATP + OxATP   | −98.5 ± 15.2   | 62.4 ± 8.4     | 3186 ± 50.3     | 34 |
| α,β-mATP        | −109.6 ± 31.1  | 70.4 ± 11.9    | 449.3 ± 53.4    | 19 |
| BzATP + 0 Ca2+  | −216.7 ± 57.8  | 13.8 ± 18.4    | 940.4 ± 108.8   | 13 |

*p > 0.1.
*p > 0.5.
*p < 0.05.

In 7 neurons in which BzATP triggered either a clear (Fig. 6A, top, n = 3) or a nearly undetectable tonic current (Fig. 6A, top, n = 4) when a second BzATP challenge was performed in low extracellular Ca2+, the amplitude of the tonic current was drastically increased (Fig. 6, A and A1, bottom, and B, left). The increase in background noise, which is always associated with the tonic current, was similarly enhanced (Fig. 6B, right). In contrast, the kinetic features of SICs evoked by BzATP in low Ca2+ are unchanged with respect to SICs evoked by BzATP in normal Ca2+ (Table 1). Because of the fact that BzATP in low Ca2+ triggered a large increase in the background noise of the trace, SICs of small amplitude were lost in the noise, and thus, only SICs of larger amplitude could be analyzed. As such, the mean amplitude of SICs evoked by BzATP in low Ca2+ results are higher than the mean amplitude of SICs evoked by BzATP in normal Ca2+.

Slice perfusion for 3–4 min with low Ca2+ in the absence of BzATP failed to evoke either a significant tonic current or an increase in noise amplitude (n = 21; Fig. 6A, and B). As we showed previously (11), slice perfusion with low Ca2+ induces Ca2+ oscillations in astrocytes and, thus, activates the Ca2+-dependent release of glutamate in these cells that leads to the generation of SICs in CA1 pyramidal neurons. Several SICs can indeed be detected in neurons upon low Ca2+ stimulation (Fig. 6A), D-AP5 (50–100 μM) reversibly abolished the tonic current evoked by application of BzATP in low Ca2+, demonstrating that it is entirely due to glutamate-mediated activation of NMDA receptors (Fig. 6C). In the presence of the P2X7R...
antagonist BBG (2 μM), BzATP application in low Ca^{2+} evoked a negligible tonic current and a small increase in background noise in 6 of 12 neurons (50%; Fig. 6D). Finally, application in low Ca^{2+} of the weak P2X-R agonist αβ-mATP triggered in 4 of 6 neurons (67%) a tonic current of negligible amplitude compared with that triggered by BzATP under the same experimental conditions (not shown).

The reverse operation of glutamate transporters and opening of hemichannels are not involved in the BzATP-induced release of glutamate, which generates the tonic current (Fig. 7). Indeed, in the presence of the glutamate transporter inhibitor TBOA (31) or of the hemichannel antagonist carbenoxolone (100 μM, Fig. 7B (32, 33)) both the amplitude of the tonic current and the increase in background noise triggered by 100 μM BzATP in low Ca^{2+} are unaffected (p > 0.4). The inhibition of glutamate uptake by TBOA results in an increase in the concentration of extracellular glutamate that, in the absence of BzATP stimulation, results in a steady state, inward current (Fig. 7A, bottom trace) with a mean amplitude of $-186.0 \pm 56.1 \text{ pA} (n = 3)$. Taken together, these observations indicate that activation of the P2X-R or of a P2X-like receptor in hippocampal slices results in the release of glutamate that mediates the tonic current.
BzATP activates [Ca\(^{2+}\)], elevations in astrocytes (34) that are known to trigger a Ca\(^{2+}\)-dependent release of glutamate from these cells (3, 5). The larger amplitude of the tonic current evoked by BzATP in low Ca\(^{2+}\) with respect to the amplitude of the tonic current evoked by BzATP in normal Ca\(^{2+}\) might, thus, be due to a higher number of astrocytes activated by BzATP in low Ca\(^{2+}\). In contrast with this hypothesis, we found that the number of astrocytes displaying [Ca\(^{2+}\)] elevations in response to BzATP (24 of 27; 89 ± 7%; n = 3) was similar to that observed after BzATP application in low Ca\(^{2+}\) (23 of 27; 84 ± 5%; n = 3, p > 0.5). Given that BzATP can also activate, besides various P2XRs, the P2Y1R (25), BzATP may act also on this metabotropic receptor to trigger Ca\(^{2+}\) elevations in astrocytes. ATP-mediated Ca\(^{2+}\) responses in stratum radiatum astrocytes.
BzATP Triggers in Astrocytes a Large Depolarization Mediated by a P2X7-like Receptor—Murine astrocytes in culture have been shown to massively release glutamate through P2X, R openings (16). To test whether functional P2X7Rs are also expressed in situ astrocytes and if they are involved in the generation of the tonic current, we performed patch clamp recordings from stratum radiatum astrocytes. In each of the cells tested (n = 11), BzATP (100 μM) evoked a slowly developing inward current (mean time to peak, 102 ± 12 s) that was greatly potentiated when BzATP was applied in low Ca2+ (Fig. 8, A and B). The current evoked by BzATP under both normal and low Ca2+ concentrations was almost abolished in the presence of BBG (Fig. 8B).

To directly test the hypothesis that BzATP opens in the astrocytic membrane a channel permeable to large molecules, such as a P2X7-like receptor, we applied voltage steps to whole-cell recorded astrocytes. We found that in all astrocytes tested (n = 5), BzATP application reversibly shifted the reversal potential (Erev) of the whole-cell I-V relationship toward more positive voltages with respect to basal conditions (Fig. 8, C and E). This shift was prevented in astrocytes from slices incubated with BBG (Fig. 8D, E, n = 4). Surprisingly, the overall membrane conductance was found to be unchanged upon BzATP application and to decrease drastically after BzATP washout (Fig. 8, C and F).

Given the low input resistance of the astrocytes and the huge current induced by BzATP, the voltage clamp measurements described above are affected by inadequate clamping of the astrocytic membrane to the imposed voltage. To address this concern we repeated the experiments in current clamp configuration. In all the astrocytes recorded (n = 4), BzATP evoked a slowly increasing depolarization of the astrocytic membrane (Fig. 8, G and H) which, as in the previous voltage-clamp experiments, is greatly potentiated when BzATP is applied in low external Ca2+ and almost completely blocked by BBG (Fig. 8H).

Activation of glutamate receptors by glutamate released from either astrocytes or neurons upon BzATP stimulation is not involved in the BzATP-induced inward current in astrocytes. Indeed, in the presence of 10 μM NBQX, 100 μM LY367385, and 50 μM 2-methyl-6- (phenylethyl)-pyridine hydrochloride, antagonists of the AMPA receptor, metabotropic glutamate receptor (mGluR) 1 and mGluR5, respectively, BzATP triggered in astrocytes an inward current of unchanged amplitude (~509.0 ± 104.3 pA, n = 11, and ~552.8 ± 121.2 pA, n = 4, in the absence and in presence of the antagonists, respectively; p > 0.5). The BzATP-induced inward current was also unaffected in the presence of 50 μM D-AP5, antagonist of the NMDA receptor (mean amplitude: ~561.7 ± 136.5 pA, n = 3, p > 0.5).

If the large inward current induced by BzATP in the astrocytic membrane is due to a large pore-forming purinergic receptor, it may allow the entrance of molecules of high molecular weight. When BzATP was applied in the virtual absence of external Ca2+, a significant uptake of LY was detected in a subpopulation of astrocytes from the CA1 region (80 of 238, 29 ± 4%; n = 8) (Fig. 9, C, cells 2–5 and E). Electrophysiological characterization of dye-loaded cells demonstrates that these cells display the passive membrane properties typical of astrocytes (n = 3, Fig. 9E).

In patch clamp experiments the BzATP-induced response triggered in low Ca2+ was observed in all astrocytes recorded, whereas BzATP in low Ca2+ induced loading of LY only in a subpopulation of these cells. We suggest that the higher sensitivity of patch clamp with respect to the dye uptake technique may account for this discrepancy. The dye uptake in astrocytes was inhibited in the presence of BBG (Fig. 9E). A BBG-sensitive dye uptake was also detected in a subpopulation of neurons from the CA3 region (71 of 168, 40 ± 5%; n = 4, Fig. 9, D and F).

After BzATP incubation, a few pyramidal neurons were also stained with LY in CA1 region. However, since the number of stained CA1 neurons from control slices was similar (Fig. 9G), the dye uptake in these neurons was independent of BzATP action and possibly reflects membrane damage after the slicing procedures.

Taken together, data from electrophysiological and dye uptake experiments support the view that a pore-forming receptor, such as the P2X7-R or a P2X7-like receptor, possibly expressed in astrocytes as well as in neurons, can open and either directly or indirectly accounts for the release of glutamate that contributes to the generation of the tonic current in CA1 neurons, in particular under low Ca2+ conditions.

DISCUSSION

In the present study we found that activation of purinergic receptors in the hippocampus activates two separate pathways of glutamate release that trigger two distinct responses in CA1 pyramidal neurons. The first pathway depends on activation of purinergic receptors in astrocytes other the P2X7R and mediates the episodic SIC in neurons. The second pathway depends on activation of a P2X7-like receptor, expressed in either astrocytes and neurons, and mediates the sustained tonic current in neurons.

The episodic SIC was previously demonstrated to be due to Ca2+-dependent glutamate release from astrocytes and to represent a form of glutamate-mediated astrocyte-to-neuron signaling that promotes synchronous activity of CA1 pyramidal neurons (10, 11). SICs evoked by BzATP, a powerful although unspecific agonist of the P2X7-R, were shown here not to be affected by the P2X7-R antagonists BBG and OxATP (24, 26, 27). This finding rules out the possibility that BzATP acts on a P2X7-R to trigger the release of glutamate-mediating neuronal SICs. Beside the P2X7-R, BzATP is known to stimulate most of the other P2 receptors, although with different potencies (24, 25, 36). BzATP can also activate metabotropic P2Y2Rs (25), including the P2Y7-R that in mouse stratum radiatum astrocytes mediates Ca2+ elevations triggered by ATP (35). Activation by BzATP of both Ca2+-permeable P2XRs (37) and metabotropic P2YRs can, thus, lead to [Ca2+]i elevations in the astrocytes and triggers the Ca2+-dependent glutamate release pathway that mediates SICs. Our observations that α,β-mATP does not effectively activate either P2X-Rs or P2Y2Rs (24, 25) also evoke SIC support for the conclusion that activation of a P2XR other than the P2X7-R is sufficient to trigger the release of glutamate from astrocytes that evoke neuronal SICs. The poor specificity of the available pharmacological tools for studying the different purinergic receptors hampers, however, a clear identification of the distinct P2XR type that mediates SICs.

The tonic current, which was always associated with a noise increase in the current trace, was found to be mediated by a purinergic receptor different from the receptor that mediated SICs. The following observations suggest that the P2X7-R is a plausible candidate. (i) In contrast to SICs, the tonic current was sensitive to both BBG and OxATP; (ii) α,β-mATP, which in contrast to BzATP weakly activated P2X7-Rs (25, 37, 38), evoked SICs but failed to trigger the tonic current; (iii) the amplitude of BzATP-induced tonic current was greatly amplified in low extracellular Ca2+, a condition that enhances P2X7-R openings (17, 37); (iv) the large amplitude tonic current evoked by BzATP in low extracellular Ca2+ was almost completely abolished in slices incubated with BBG; (v) in low Ca2+, the weak P2X7-R agonist α,β-mATP triggered a tonic current of negligible amplitude compared with that triggered by BzATP under the same experimental conditions.

It is worth underlying that lowering extracellular Ca2+ results in a
2–3-fold increase in the amplitude of the NMDA receptor-mediated current and reduces inactivation of this receptor (39, 40). Although this change in NMDA receptor intrinsic properties may contribute to increase the amplitude of the tonic current triggered by BzATP-induced release of glutamate in low Ca²⁺, it is highly unlikely that it can fully account for the observed 25-fold increase in the tonic current amplitude.

Besides enhancing the opening of P2X₇ receptors, lowering extracellular Ca²⁺ may also enhance hemichannel openings. Under these conditions, cytoplasmic glutamates of astrocytes have been reported to exit through these large conductance channels along its concentration gradient (41). Our results suggest that this release pathway does not contribute significantly to the observed 25-fold increase in the tonic current amplitude.

As to the cells that express the P2X₇ receptor, astrocytes are good candidates. By reverse transcription-PCR analysis and Western blotting, astrocytes in culture have been shown to express, besides various P2YRs, all the P2XR except the P2X₆R (34). Immunocytochemical studies in rat brain slices also revealed various P2XR, including the P2X₇R, to be expressed in hippocampal astrocytes (42). In murine cultured astrocytes, BzATP has been reported to trigger a massive, slow efflux of glutamate into the extracellular space, suggesting that these cultured cells express functional P2X₇R (16). The following results from the present study are compatible with a functional expression of P2X₇R in hippocampal astrocytes in situ. (i) In all astrocytes recorded, in either voltage or current clamp configuration, a large amplitude inward current and a depolarization, respectively, slowly developed upon BzATP application; (ii) both responses were amplified in low Ca²⁺, and they were almost completely blocked in slices incubated with BBG; (iii) in the presence of specific antagonists of AMPA, NMDA, and type I metabotropic glutamate receptors, BzATP-induced current was unchanged. Therefore, activation of these glutamate receptors in astrocytes by BzATP-induced release of glutamate does not contribute significantly to the BzATP-

FIGURE 9. Stratum radiatum astrocytes and CA3 neurons are permeabilized by BzATP stimulation with low extracellular Ca²⁺. A–C, differential interference contrast and corresponding fluorescence images of CA1 region under control conditions (A), in the presence of BzATP (B), and in the presence of BzATP and low Ca²⁺ (C). Images are the averages of 20 consecutive acquisitions. Bar, 20 μm. D, differential interference contrast and corresponding fluorescence images of CA3 region in the presence of BzATP and low Ca²⁺. Bar, 20 μm. E–G, average percentage of positive stratum radiatum astrocytes (E, see the inset for passive membrane potential changes induced by current injections in whole-cell recording from a dye-loaded cell; V_m = −61 mV), CA3 (F), and CA1 (G) neurons under the different experimental conditions. The numbers close to the bars are the number of performed experiments. *, p < 0.05; **, p < 0.01.
induced current. These data also argue against the possibility that an increase in the extracellular concentration of K⁺, which may derive from depolarizing neurons after activation by astrocytic glutamate of AMPA and NMDARs, is involved in BzATP-induced currents in astrocytes; (iv) in a fluorescent dye permeability assay, a BBG-sensitive uptake of lucifer yellow was detected in astrocytes upon stimulation with BzATP in low Ca²⁺. Dye loading was undetectable in slices incubated with normal Ca²⁺ as well as in slices incubated with normal Ca²⁺ and BzATP.

According to these observations, BzATP application should open in the astrocytic membrane a non-selective channel, such as a P2X-R that in the large pore configuration allows cations such as K⁺, Na⁺, and Ca²⁺ and anions such as Cl⁻ and glutamate to flow through the membrane according to their electrochemical gradient, ultimately driving the reversal potential of the whole-cell I-V relationship to more positive values. During BzATP application, the I-V curve was indeed observed to shift significantly along the voltage axis in the depolarizing direction. Although this result is compatible with the opening of a P2X-like receptor, the overall membrane conductance was surprisingly found to be unchanged upon BzATP application and to decrease drastically after BzATP washout. A possible interpretation of this contradictory result is that the expected increase in membrane conductance due to P2X-like receptor openings is masked by the overall decrease in membrane conductance that is triggered by BzATP and becomes evident only after BzATP washout.

Taken together, results obtained are compatible with the view that P2X-R openings in astrocytes lead to formation of a large conducting pore that allows the uptake of molecules of relatively large molecular weight. Further experiments are necessary to provide conclusive evidence on this issue.

BzATP applied in normal Ca²⁺ evoked a tonic current response in 35% of neurons, whereas it evoked an inward current in all astrocytes tested. A plausible hypothesis for this discrepancy is that the variability of the neuronal response reflects the non-uniform organization of astrocyte-neuron contacts in the hippocampus (43). Neurons unresponsive to BzATP may be located far away from astrocyte processes and display a tonic current only when the release of glutamate from astrocytes is greatly potentiated, as in the case of BzATP applied in low Ca²⁺. Indeed, when BzATP was applied in low Ca²⁺, the tonic current response was observed in all neurons. Noteworthy is that the same neurons that did not display a detectable response to a first BzATP challenge in normal Ca²⁺ displayed a clear tonic current in response when a second BzATP challenge was applied in low Ca²⁺.

In the hippocampus, besides astrocytes, CA3 pyramidal neurons as well as neurons from the dentate gyrus have been reported to express the P2X-R (44–46). Although this point was not addressed in detail here, we observed that in slices incubated with low Ca²⁺ and BzATP, a BBG-sensitive uptake of the dye was detected in a subpopulation of CA3 neurons. Glutamate released through P2X-Rs expressed on CA3 axon terminals of Schaffer collateral pathway may, thus, contribute to the tonic current that we recorded from CA1 neurons.

As to the functional role that glutamate derived from P2X-R activation may have, it has to be taken into account that the endogenous agonist of purinergic receptors, ATP, is a weak P2X-R agonist. It is, thus, unlikely that ATP released under physiological conditions from either axon terminals of Schaffer collaterals (47) and/or astrocytes (35, 48) can reach the high extracellular concentration that is necessary to activate the P2X-R. Most importantly however, such a high concentration may be reached under pathological conditions, such as ischemia and brain trauma (49). The consequent activation of P2X-Rs may result in a massive glutamate release and contributes to increase the extracellular glutamate to the abnormal levels that cause excitotoxic cell death (50). Consistent with this hypothesis, P2X-R inhibition has been recently found to improve the functional recovery and to decrease the death of motoneurons after acute spinal cord injury in rats (51).

It has to be underlined that the expression of the P2X-R in brain cells other than activated microglia (17, 18) is highly debated (52), and conflicting results have been reported. Although previous studies provide indications for the presence of the P2X-R in both astrocytes (42) and neurons from the CA1, CA3, and dentate gyrus region (44, 45, 53, 54), more recent studies cast serious doubts on this conclusion (55, 56).

The results reported in the present study are compatible with the functional expression of the P2X-R in hippocampal cells, either astrocytes and/or neurons. However, also due to the lack of highly specific agonists and antagonists for the different purinergic receptors, we can only suggest that the release of glutamate which triggers the tonic current is due to the activation of the P2X-R or a P2X-like receptor. Indeed, other P2XRs, such as the P2X7R and the P2X7-R, that have been suggested to result in a progressive formation of a large conducting pore (57, 58) are expressed in stratum radiatum astrocytes (42) and may, thus, be involved in the generation of the tonic current.

In conclusion, the results here reported clearly demonstrate that activation of different purinergic receptors in the hippocampus mediates two types of glutamate release, each evoking a distinct response in CA1 pyramidal neurons. In the first type of release, the P2X-R is not involved. This type of glutamate release from astrocytes evokes in CA1 pyramidal neurons transient NMDAR-mediated responses (SICs) that represent a hallmark of astrocyte-to-neuron communication (11). In the second type of release, the P2X-R or a P2X-like receptor, may be involved. This release triggers a slowly developing, tonic current and contributes to increase the concentration of extracellular glutamate. This event is potentiated under non-physiological conditions, raising the possibility that it contributes to the excitotoxic action of glutamate in the brain.

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P2X$_7$ Receptor-mediated Glutamate Release

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