Ecto-Nucleotidases and Nucleoside Transporters Mediate Activation of Adenosine Receptors on Hippocampal Mossy Fibers by P2X_7 Receptor Agonist 2′-3′-O-(4-Benzoylbenzoyl)-ATP

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The ionotropic and cytolytic P2X receptor is typically found on immune cells, where it is involved in the release of cytokines. Recently, P2X receptors were reported to be localized to presynaptic nerve terminals and to modulate transmitter release. In the present study, we reassessed this unexpected role of P2X receptors at hippocampal mossy fiber–CA3 synapses. In agreement with previous findings, the ionotropic and cytolytic P2X7 receptor is typically found on immune cells, mediating the release of cytokines (Le Feuvre et al., 2002), and is involved in the induction of apoptosis (Brough et al., 2002). With the exception of retina and auditory spiral ganglion cells (Chizh and Illes, 2001; Khakh, 2001), the role of P2X receptors in neurotransmission and modulation of transmitter release has been described at certain central synapses (Edwards et al., 1992; Nieber et al., 1997; Khakh and Henderson, 1998; Pankratov et al., 1998; Khakh et al., 1999, 2003; Norenberg and Illes, 2000; Kato and Shigetomi, 2001; North, 2002). In contrast to P2X1–P2X6, the expression and function of P2X7 in neuronal and non-neuronal tissues (for review, see North, 2002) are still unclear (Norenberg and Illes, 2000; Chizh and Illes, 2001). P2X7 receptors are cationic channels as well, but they display several differences from the other subtypes. P2X7 receptors form large diameter cytolytic pores in the continuous presynaptic c-terminal domain enables them to couple to various intracellular signaling cascades (Denlinger et al., 2001). Typically, P2X7 receptors are located on immune cells, mediating the release of cytokines (Le Feuvre et al., 2002), and are involved in the induction of cell death (Brough et al., 2002). With the exception of retina and auditory spiral ganglion cells (Brandle et al., 1998, 1999), P2X7 mRNA and protein have not been found in adult rat brain parenchyma (Collo et al., 1997).

It was reported recently, however, that P2X7 subunits are

Introduction

In addition to its classic role as an intracellular energy source, ATP is now recognized as an extracellular signaling molecule that acts at two distinct classes of receptors: metabotropic P2Y and ionotropic P2X receptors (Ralevic and Burnstock, 1998). P2X receptors are nonselective cation channels, forming a family of at least seven subunits (P2X1–P2X7), and are expressed in various neuronal and non-neuronal tissues (for review, see North, 2002). The most unequivocal functional role of neuronal P2X receptors (P2X1–P2X7) is the processing of pain as presynaptic and postsynaptic receptors in spinal cord and dorsal root ganglion parenchyma (Collo et al., 1997).

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localized on presynaptic terminals and modulate transmitter release onto hippocampal and spinal cord neurons (Deuchars et al., 2001; Armstrong et al., 2002; Sperlagh et al., 2002). The presence of Ca$$^{2+}$$-permeable P2X$$\_$$ receptors on hippocampal mossy fiber terminals (Armstrong et al., 2002; Sperlagh et al., 2002) would be particularly intriguing, because mossy fiber synapses display several forms of short and long-term synaptic plasticity that depend on the accumulation of presynaptic Ca$$^{2+}$$ (Castillo et al., 1994; Regehr et al., 1994; Salin et al., 1996; Dietrich et al., 2003). Because the endogenous ligand ATP is released by hippocampal slices (Wieraszko et al., 1989; Cunha et al., 1996; Inoue, 1998), presynaptic P2X$$\_$$ receptors could exert an activity-dependent modulation of mossy fiber synaptic transmission. So far, however, results are conflicting, because on application of the (non-selective) P2X$$\_$$ agonist 2'-3'-O-(4-benzoylbenzoyl)-adenosine-5'-triphosphate (BzATP) (North, 2002), one group reported an increase (Sperlagh et al., 2002) and another group reported an inhibition (Armstrong et al., 2002) of transmitter release.

A potential problem when studying the functional role of P2X receptors in native tissue such as brain slices is that many of the commonly used agonists, ATP and analogs, are subject to rapid breakdown to adenosine by extracellular enzymes called "ecto-nucleotidases" (Dunwiddie et al., 1997; Cunha et al., 1998; Zimmermann and Braun, 1999; Zimmermann, 2000). These nucleotidases lead to formation of sufficient levels of adenosine to activate presynaptic and postsynaptic adenosine A1 receptors after application of even micromolar concentrations of ATP onto hippocampal slices (Dunwiddie et al., 1997; Cunha et al., 1998). It has not been determined whether the commonly used P2X agonist, BzATP, is catalyzed by ecto-nucleotidases as well. Therefore, in the present study we reassessed the suggested role of P2X$$\_$$ receptors in the modulation of transmitter release at the mossy fiber–CA3 synapse. Although we confirm that application of BzATP produces a pronounced inhibition of mossy fiber synaptic transmission, our conclusions regarding the involved mechanisms are completely different from those published previously (Armstrong et al., 2002). Our results suggest that BzATP is catalyzed by hippocampal slices and that the observed effects of BzATP are caused by activation of adenosine receptors and require enzymatic conversion by ecto-nucleotidases to Bz-adenosine and a heteroexchange with cellular adenosine via nucleoside transporters.

Materials and Methods

Slice preparation. Hippocampal slices (400 µm thick) were prepared from 20- to 40-d-old Wistar rats (Charles River, Munich, Germany) or adult mice (see below). Animals were anesthetized with chloroform and allowed to recover at 34°C and 95% O2 and 5% CO2 mixture. Horizontal hippocampal slices were cut with a vibrating blade microtome (Leica Microsystems, Nussloch, Germany) in artificial cerebral spinal fluid (ACSF) containing (in mM): 124 NaCl, 2.5 KCl, 1.25 NaH2PO4, 7 MgSO4, 0.5 CaCl2, 25 NaHCO3, 25 glucose, 75 sucrose, gassed with a 95% O2 and 5% CO2 mixture. Horizontal hippocampal slices were cut with a vibrating blade microtome (Leica Microsystems, Nussloch, Germany). Slices were quickly transferred to an interface incubation chamber and allowed to recover at 34°C for 30 min in solution of a composition similar to that used for the preparation. Slices were then stored at room temperature in artificial CSF (ACSF) containing (in mM): 124 NaCl, 3 KCl, 1.25 NaH2PO4, 2 MgSO4, 2 CaCl2, 26 NaHCO3, 10 glucose, pH 7.4, osmolality 300 mosm, pH 7.4, gassed with a 95% O2 and 5% CO2 mixture. At least 1 hr after preparation, individual slices were transferred to a submerged recording chamber mounted on the stage of an upright Nikon microscope (Nikon E600FN) and superfused continuously (2 ml/min) with gassed ACSF. Drugs were added to this superfusion solution. In some cases (see Results), slices were preincubated for 45–60 min with drugs [inosine 5’-triphosphate trisodium salt (ITP), 4-[5-(4-fluorophenyl)-2-(4-methylsulfonyl)phenyl]-1H-imidazol-4-yl]pyridine (SB 203580), 6-N,N-diethyl-β-γ-dibromomethylenecarbamoyl-adenosine-5’-triphosphate trisodium salt (ARL 67156), concanavalin A]. During that time, slices were kept in a small volume gassed interface-like chamber in the presence of the drug. For control purposes, slices were kept under the same conditions but without the drug addition. Because the responses obtained from slices without preincubation and from slices preincubated without drug were not different, data were pooled and taken as a common control group.

Electrophysiological voltage-clamp recordings. Whole-cell voltage-clamp experiments were performed using patch pipettes pulled from borosilicate glass on a vertical puller (model PP-830, Narishige, Tokyo, Japan). Electrodes had a resistance of 4–5 MΩ when filled with our internal solution containing (in mM): 125 potassium gluconate, 0.5 HEPES, 2 MgCl2, 23 KCl, 3 NaCl, pH adjusted to 7.3 with KOH, osmolality 280–290 mosm/kg. Voltage was corrected for the liquid junction potential by offsetting the amplifier to −6 mV before seal formation. Tetrodotoxin (TTX) (500 nm) and kynurenic acid (2 mM) were included in the standard external solution (above-mentioned ACSF). Pyramidal cells were located visually in the CA1 or CA3 somal layer using infrared differential interference contrast videomicroscopy. Cells were voltage clamped at −65 mV with a patch clamp L/M-EP 7 amplifier (HEKA, Lambrecht-Pfalz, Germany). Holding current was averaged over several hundred milliseconds every 2 sec. The access resistance was determined from the current response to a −5 mV hyperpolarizing voltage command step every 2 sec and was typically 10–25 MΩ. The responses were low-pass filtered at 1–2 kHz, digitized with a sampling frequency of 20 kHz (TTC-16, HEKA), and analyzed using Igor Pro software (WaveMetrics, Lake Oswego, OR).

Extracellular electrophysiological recordings. Extracellular recordings of the field EPSPs (fEPSPs) were made using glass microelectrodes (2–4 MΩ) filled with ACSF and placed under visual guidance in stratum lucidum of the CA3 region. Monopolar stimulation (rectangular pulses of 0.1 msec applied once every 30 sec) of mossy fibers was delivered through a glass electrode (resistance 1 MΩ) placed in stratum lucidum. The mossy fiber origin of fEPSPs was verified at the end of each experiment by application of 25,2'R,3'R-2-(2',3'-dicarboxyethyl)pyridine (DCG-IV) (1 µM). The experiments were included only if the inhibition produced by DCG-IV was >75%. fEPSPs were recorded with a SEC-05L amplifier (npi Electronics, Tamm, Germany), filtered at 3 kHz, and digitized at 20 kHz. The peak amplitudes of the fEPSPs were analyzed using Igor Pro software, and the responses were normalized to average value calculated 10–20 min before BzATP application and expressed in percentage.

In vitro analysis of hydrolysis of ATP analogs. Catabolism of ATP, BzATP, αβ-methylene-ATP (βM-ATP), and αβ-MeADP was analyzed by incubating these substrates with enzymes and measuring the amount of inorganic phosphate (P$_i$) released. The phosphate concentration was determined spectrophotometrically using the P$_i$ Per Phosphate Assay kit (P-22061, Molecular Probes, Leiden, The Netherlands) according to the protocol provided by the manufacturer. We used commercially available purified members of the nucleotide triphosphatase (NTPDase) family and ecto-5’-nucleotidase (5NTase) (apryase, E.C. 3.6.1.5 and 5’-nucleotidase, E.C. 3.1.3.5; Sigma, Deisenhofen, Germany) because it is known that those enzymatic activities are present in hippocampal slices (Cunha et al., 1992, 1998, 2000; Braun et al., 2000; Boeck et al., 2002; Bruno et al., 2002). To compare the amount of P$_i$ released from the various substrates (see Fig. 5A), we proceeded as follows: 250 µl of reaction solution containing 100 µM Tris–HCl, pH 7.5, 60 µM substrate, 2 mM CaCl$_2$, and 0.5 µM of each enzyme was incubated for 10 min at 35°C. After that time, 250 µl of the detector kit reagents was added. Over the next 45 min, the free phosphates initiated a chain of enzymatic reactions (provided by the kit) that finally converts Amplex Red to the fluorescent resorufin. Resorufin absorption was measured at 560 nm. In each run we included two to five vials containing standard P$_i$ concentrations of 20–100 µM and an "empty" vial containing only Tris and CaCl$_2$. In a subset of experiments, we also included "no-substrate" and "no-enzyme" controls that produced essentially the same absorption as the empty vials (some no-enzyme controls are shown in Fig. 5A). Values of Δabsorption (Δabs) were measured as the difference between the absorption of the empty and the test vial. In the P$_i$ concentration...
range of interest, Δabs was linearly related to P, concentration, and the amount of P, released from the substrates was determined by interpolation of Δabs values of the P, standards. To compare the apparent affinity of enzymes for ATP and BzATP (see Fig. 5B), we decreased the enzyme activity 10-fold and CaCl₂ concentration 100-fold. This slowed the reaction and made it possible to compare the initial accumulation of phosphate (1 min) during nearly constant substrate concentration. Three vials with BzATP and three vials with ATP were started together with a control vial by adding the substrates. The reaction in the different vials was stopped after 1, 3, and 6 min at 35°C by cooling down and adding the reagents of the detector kit that were supplemented for this experiment by 10 μl EDFTA. The control vial was identical to the others (either ATP or BzATP) but the EDFTA–kit reagent mixture was added at time 0. Δabs was calculated as the difference between the test and the control vial and normalized for each run on the 6 min value of ATP.

Confocal calcium imaging. For imaging of intraterminal Ca²⁺ transients, mossy fibers were loaded with the high-affinity Ca²⁺ indicator Oregon Green acetoxymethyl ester ( Molecular Probes) as described previously for other indicators (Dietrich et al., 2003). Briefly, a small amount of Oregon Green acetoxymethyl ester was dissolved in DMSO containing 20% pluronic acid and pressure injected in the stratum lucidum of hippocampal slices maintained in an interface chamber. After a period of at least 60 min to allow the indicator to diffuse along the presynaptic fibers, individual slices were placed in a submerged chamber in which ITP (2 mM) was added to ACSF gassed with a 95% O₂ and 5% CO₂ mixture. Slices were transferred to the stage of a confocal microscope (Zeiss LSM5 Pascal, Axioskop FS 2) 45–60 min later and superfused with ACSF containing 2 mM ITP and Trolox (15 mg/ml) at room temperature. To ensure that mossy fibers were viable, a stimulating electrode filled with potassium conductance

Results

BzATP-induced depression of mossy fiber synaptic transmission is reversed by an adenosine A₁ receptor antagonist

In the first series of experiments, we investigated the sensitivity of mossy fiber synaptic transmission to application of the P₂X₇ agonist BzATP (Ralevic and Burnstock, 1998; Khakh et al., 2001). BzATP (65 μM) inhibited mossy fiber IEPSPs by 41 ± 4% (n = 4) (Fig.1A, C), similar to a previous report (Armstrong et al., 2002). Armstrong et al. (2002) assumed that this effect was caused by the activation of P₂X₇ receptors on mossy fiber terminals by BzATP; however, because mossy fiber transmission is also sensitive to the action of adenosine via presynaptic inhibitory A₁ receptors (Okada and Ozawa, 1980; Scanziani et al., 1992) and hippocampal slices can potently convert ATP and various analogs to adenosine (Khakh et al., 2001), we asked whether the depression induced by BzATP could be reversed by an A₁ agonist. Indeed, the specific A₁ receptor antagonist 8-cyclopentyl-1,3-dipropylxanthine (DPCPX) (Lohse et al., 1987), reversed the effect of BzATP (Fig. 1A). Similarly, the BzATP-induced depression was completely abolished if DPCPX (1 μM) was preappllied (Fig. 1B, C) (p < 0.001). It should be noted, however, that 1 μM DPCPX possibly blocks A₂B receptors as well (see Discussion).

BzATP leads to activation of an A₁ receptor-coupled potassium conductance

If application of BzATP leads to the activation of presynaptic A₁ receptors on mossy fiber terminals, e.g., after enzymatic conver-
sion, then it should also lead to activation of postsynaptic adenosine receptors. Postsynaptic adenosine receptors on hippocampal pyramidal cells are coupled to G-protein-gated inwardly rectifying potassium (GIRK) channels (Luscher et al., 1997). Indeed, during whole-cell voltage-clamp recordings of CA3 pyramidal neurons, 65 \( \mu \text{M} \) BzATP-induced an outward current of 26 \( \pm \) 2 pA (26 \( \pm \) 2 mV; \( n = 2 \); data not shown). Because the enzymatic conversion of ATP to adenosine and the consecutive activation of adenosine-coupled GIRK channels has been characterized in great detail in CA1 pyramidal cells (Dunwiddie et al., 1997; Luscher et al., 1997; Takigawa and Alzheimer, 2002), we further studied the effects of BzATP in the CA1 region. In CA1 pyramidal cells, bath application of BzATP also elicited a clear concentration-dependent outward current of 5 \( \pm \) 0.7 pA (26 \( \pm \) 2 mV; \( n = 8 \)) and 14 \( \pm \) 3 pA (30 \( \pm \) 2 mV; \( n = 14 \)) at 20 and 65 \( \mu \text{M} \), respectively (Fig. 2A, middle panels). Currents recorded in response to voltage ramps before and during superfusion of the drug revealed that the BzATP current reversed around the potassium equilibrium potential (26 \( \pm \) 2 mV; \( n = 8 \)) (Fig. 2C, left panel), as expected for GIRK channels (Luscher et al., 1997). In agreement with a previous report (Dunwiddie et al., 1997), the observed current–response was mimicked by ATP and adenosine. ATP induced a concentration-dependent outward current of 12 \( \pm \) 1.6 pA (30 \( \mu \text{M}; n = 12 \)) and 16 \( \pm \) 5 pA (100 \( \mu \text{M}; n = 5 \)) (26 \( \pm \) 2 mV; \( n = 3 \)) that reversed at \(-90 \pm 4 \text{ mV} \) (Fig. 2A–C, middle panels). Similarly, adenosine elicited an outward current of 9 \( \pm \) 3 pA (26 \( \pm \) 2 mV; \( n = 9 \)) and 12 \( \pm \) 1 pA (26 \( \pm \) 2 mV; \( n = 14 \)) at 30 and 100 \( \mu \text{M} \), respectively (26 \( \pm \) 2 mV; \( n = 3 \)) (Fig. 2A–C, right panels).
adenosine deaminase (2 U/ml) resulted in a complete reversal of the outward current evoked by osine (Fig. 3). Complete reversal of the current evoked either by BzATP or by adenosine (and 2’-deoxyinosine) (Cristalli et al., 2001) reversed the outward current caused by BzATP (Fig. 3). Importantly, adenosine was still active, showing that the function of A1 receptors is not affected by ARL 67156 (Fig. 4A) (n = 4). Likewise, when the E-NTPDase substrate ITP was added in excess (2 mM) to competitively reduce the hydrolysis, the current activated by BzATP but not that activated by adenosine was blocked (Fig. 4B, D) (n = 5). The final step of catabolism of ATP by ecto-nucleotidases is the conversion of AMP to adenosine. This step is catalyzed by ecto-SNTase and can be inhibited by concanavalin A (Stefanovic et al., 1975; Zygoicz et al., 1977; Westfall et al., 2002). To further substantiate the necessity of the ecto-nucleotidase pathway for BzATP to be active, we confirmed that concanavalin A blocked BzATP but not adenosine-induced currents (Fig. 4C, D) (n = 4). 

Coperfusion of an adenosine-specific deaminase (Cristalli et al., 2001) reversed the outward current caused by BzATP (Fig. 3). This indicates that when BzATP is superfused, it is adenosine that activates GIRK-coupled A1 receptors; however, in contrast to the hydrolyzation of ATP, the hydrolyzation of BzATP should only give rise to Bz-adenosine and not adenosine itself (see Discussion). We therefore asked whether nucleoside transporters (NTrans) could hetero-exchange extracellular Bz-adenosine for adenosine from intracellular sources (compare Fig. 9). Various types of NTrans systems are present in the brain, which is deficient of de novo synthesis of nucleotides (Pastor-Anglada et al., 1998; Lee et al., 2001). For this reason, NTrans are essential for the salvage of nucleosides for nucleotide and nucleic acid synthesis in the brain (Lee et al., 2001). Inosine is a substrate of a large number of such NTrans and has frequently been used to competitively inhibit their transport activity (Yao et al., 1997; Pastor-Anglada et al., 1998; Li et al., 2001). Indeed, outward current induced by BzATP (30 μM) was reversed when we added 2 mM inosine to the superfusion medium (n = 6) (Fig. 4E). As mentioned above, enzymatic conversion of ATP leads directly to formation of adenosine. Thus, activation of GIRK channels by ATP should not be sensitive to the inhibition of NTrans by coapplication of inosine. In support of this idea, 2 mM inosine did not decrease ATP-induced outward current (n = 6) (Fig. 4F), which also shows that the function of ecto-nucleotidases and A1 receptors is unaffected by inosine.

It has been suggested previously that the depression of mossy fiber synaptic transmission by BzATP involves activation of a MAP kinase (Armstrong et al., 2002). The key experiment that led the authors to that conclusion was that the presumed specific p38 MAP kinase inhibitor SB 203580 blocked BzATP-induced depression of fEPSPs, whereas it did not affect the adenosine receptor-mediated depression of synaptic transmission (Armstrong et al., 2002). There could be an alternative explanation, however, for the differential blocking effect of SB 203580: SB
BzATP is hydrolyzed in vitro by E-NTPDase and E-5NTase. A, Incubation of ATP, BzATP, α,β-MeATP, or αβ-Me-ADP with the enzymes in vitro resulted in accumulation of P_i. Note that an identical amount of P_i was released from ATP and BzATP. Without enzymes (E), only negligible amounts of P_i were detected. Incubation of αβ-MeATP yielded only a small amount of P_i. αβ-MeATP was resistant to hydrolysis. B, Time course of P_i release from ATP (■) and BzATP (□) during the incubation of these adenine nucleotides with ecto-nucleotidases. The reactions were started in parallel and stopped successively after 1, 3, and 6 min. Enzymatic activity and Ca^{2+} concentration were strongly decreased to reduce the reaction rate. After 1 min, only a small portion of substrates has been hydrolyzed, and >6 min would be required under those conditions to achieve equilibrium. Note that the initial accumulation of P_i, being proportional to the initial reaction velocity, is similar for BzATP and ATP. Absorption values were normalized in each run on the absorption value of the ATP vial that was incubated for 6 min (norm. Δabs.).
of $3.3 \pm 0.6$ nmol P$_i$ only ($n = 6$), but $\alpha\beta$-MeADP was completely resistant to enzymatic breakdown (Fig. 5A).

The above experiments demonstrate that BzATP is a substrate of NTPDase/5NTase; however, because reactions were run until steady state, it is not possible to infer from that data whether there is a preferential hydrolysis of ATP versus BzATP. As a rough test of the apparent affinity of the enzymes for ATP versus BzATP, we compared the respective initial reaction velocities. We slowed down the reaction by decreasing enzyme activity (0.2 U/ml each) and Ca$^{2+}$ concentration (0.02 mM) in the incubation buffer to minimize the reduction in initial substrate concentration. Reactions were stopped at various time points with EDTA. After 1 min reaction time, the amount of P$_i$ released from ATP and BzATP was nearly identical (Fig. 5B), suggesting that the enzymes have a similar affinity for both substrates.

Given that SB 203580 inhibits p38 MAP kinase by occupation of its ATP binding site (Young et al., 1997), we speculated that SB 203580 interferes with the binding of BzATP or ATP to E-NTPDases as well. For this reason we checked whether SB 203580 inhibits the hydrolysis of nucleotides in our in vitro assay; however, the amount of phosphate released from ATP in the presence of SB 203580 was $101 \pm 9\%$ ($n = 7$) when normalized on the amount of phosphate released in control trials run in parallel without SB 203580. Similarly the catabolism of BzATP was unchanged by the presence of SB 203580 ($97 \pm 7\%$; $n = 4$). Thus, in agreement with the above finding that adenosine- and ATP-induced currents are unaffected by SB 203580, the catabolism of nucleotides appears to be unaffected by SB 203580.

BzATP does not increase [Ca$^{2+}$]$_i$ levels in mossy fiber terminals

The data so far demonstrate that BzATP is hydrolyzed by hippocampal slices and that the reported effects of this drug can be explained by activation of $\alpha_2$ receptors instead of P2X$_2$ receptors; however, two recent studies using (the same) antibodies directed against P2X$_2$, described strong labeling of mossy fibers terminals (Armstrong et al., 2002; Sperlagh et al., 2002). Thus the question arises whether P2X$_2$ receptors can possibly be activated by BzATP when enzymatic breakdown is inhibited. Our aforementioned experiments have revealed that ITP is a useful tool to prevent the breakdown of BzATP (Fig. 4).

It is well known that P2X$_2$ receptors are Ca$^{2+}$ permeable and that in the continuous presence of the agonist (>5 min), this channel typically forms a large-conductance pore through which even molecules with a molecular weight up to 900 Da can pass (Steinberg et al., 1987; Michel et al., 1999; North, 2002). Therefore, activation of P2X$_2$ should lead to an increase of intracellular Ca$^{2+}$ concentration, and this increase has been used in many studies to assess the function of this receptor subtype (Schilling et al., 1999; Naemsch et al., 2001; James and Butt, 2002; North, 2002; Nobile et al., 2003). In the presence of ITP, we tested whether application of BzATP leads to an increase in Ca$^{2+}$ levels in mossy fiber terminals. Mossy fibers were loaded with the high-affinity Ca$^{2+}$ indicator Oregon Green–BAPTA-1-AM and imaged with confocal laser scanning microscopy (Fig. 6A). Viability of the presynaptic terminals was assessed by stimulating the fibers and obtaining a clear fluorescence increase that is caused by action potential-induced Ca$^{2+}$ entry (Fig. 6B). After that, TTX was added to the bath to reduce spontaneous activity. Application of 65 $\mu$M BzATP for 17 min did not result in any increase in resting fluorescence ($101 \pm 3\%$; $n = 4$; not significant; paired t test), indicating that there was no P2X$_2$-mediated Ca$^{2+}$ influx (Fig. 6A, middle, C, summary graph). In contrast, we could detect even a small elevation in Ca$^{2+}$ caused by a low concentration of the Ca$^{2+}$ ionophore ionomycin (130 $\pm$ 8%; $n = 4$; statistically significant; paired t test) (Fig. 6A, right, C, summary graph).

Similar Ca$^{2+}$ imaging experiments, albeit without ITP, were performed under different conditions that have been shown previously to potentiate P2X$_2$ function in other preparations (Michel et al., 1999). We reduced the concentration of extracellular divalent cations (down to 0 Mg$^{2+}$ and 1 Ca$^{2+}$), substituted sodium (up to 90%) with sucrose ($n = 5$), and elevated the recording temperature (35°C); however, we never observed a Ca$^{2+}$ increase in mossy fiber terminals on perfusion of BzATP (60–200 $\mu$M). In an additional series we incubated slices with 0.1% Lucifer
yellow and BzATP (n = 4), but again we never observed uptake of the dye into mossy fiber terminals or any other neuronal structure in the hippocampus.

**P2X7 immunoreactivity and BzATP effect on synaptic transmission in mice lacking P2X7 receptors**

As mentioned above, immunohistochemistry with commercial anti-P2X7 antibodies (Alomone Labs) revealed strong labeling of mossy fiber terminals in the hippocampus (Armstrong et al., 2002; Sperlagh et al., 2002). Using the same antibodies, we could reproduce the reported staining pattern in both mouse (Fig. 7B) and rat (data not shown) hippocampus. As illustrated in Figure 7, B and D, confocal laser scanning microscopy revealed dense immunoreactive terminals throughout the mossy fiber termination zone in the hilus and in stratum lucidum of CA3. Fainter staining was also observed throughout the hippocampus (with the exception of cell bodies). To test the specificity of antibody binding, we stained brain slices from P2X7−/− mice using the same protocol. In one strain of P2X7−/− mice, the part of the receptor that is recognized by the antibody, the C terminus (Alomone Labs), has been deleted (Solle et al., 2001). PCR analysis of DNA samples extracted from tail and hippocampal tissue confirmed the absence of the DNA sequence encoding for the C terminus in our strain of P2X7−/− mice (Fig. 7A). Still, despite the absence of this antigen, the staining of hippocampal mossy fiber terminals was completely unchanged in P2X7−/− mice (Fig. 7E). To verify this finding, we stained another strain of P2X7−/− mice in which the receptor knock-out has been performed by a frame shift (Sikora et al., 1999). Similarly, immunostaining of the mossy fiber termination zone was not altered when compared with wild-type mice (data not shown). We further investigated the subcellular distribution of antibody binding using immunogold labeling and electron microscopy (Fig. 7C). Gold grains were predominantly seen between and on the vesicles of mossy fiber boutons but encountered only rarely near the presynaptic membrane (Fig. 7C). Thus the subcellular distribution of this antigen is not compatible with a plasmalemmal receptor that can be activated by extracellular nucleotides.

To substantiate our finding that P2X7 does not contribute to BzATP-mediated depression of synaptic transmission in the mossy fiber pathway, we studied the effect of BzATP on mossy fiber fEPSPs in P2X7−/− mice (both strains). As expected, bath application of BzATP (65 μM) clearly inhibited the fEPSPs in P2X7−/− mice as in wild-type mice: fEPSP amplitudes were reduced by 44 ± 5% (Fig. 8A) (n = 3). Also, the action of BzATP was reversed by application of DPCPX (Fig. 8A), and BzATP-induced depression was completely abolished if DPCPX (1 μM) was preapplied to the slices (Fig. 8B,C) (n = 3; p < 0.005).

**Discussion**

In this study, we reassessed the suggested role of presynaptic P2X7 receptors in depressing glutamate release at the hippocampal mossy fiber–CA3 synapse (Armstrong et al., 2002). Our main findings are that there is no evidence for P2X7 receptors on mossy fiber terminals and the widely used (nonselective) P2X7 agonist BzATP leads to activation of A1 receptors via a novel, previously not recognized pathway: BzATP is enzymatically converted by ecto-nucleotidases and the product is heteroexchanged for adenosine by NTrans in hippocampal slices.

We investigated the action of BzATP in detail by monitoring the activation of GIRK channels in whole-cell voltage-clamp recordings. Enzymatic activity of ecto-nucleotidases was necessary for activation of A1-coupled GIRK channels after perfusion of BzATP (Fig. 4). This finding and our in vitro assay (Fig. 5) indicate that the phosphate groups of BzATP can be hydrolyzed by ecto-nucleotidases present in hippocampal slices. A very similar catabolism by ecto-enzymes in hippocampal slices has been described for ATP and ATP analogs (Dunwiddie et al., 1997; Cunha et al., 1998).

A number of our results demonstrate that application of BzATP onto hippocampal slices leads to activation of A1 receptors. (1) BzATP mimics the depression of mossy fiber synaptic transmission by A1 receptors (Okada and Ozawa, 1980; Scanziani et al., 1992) (Fig. 1). (2) It mimics the activation of GIRK channels on CA3 and CA1 neurons, respectively (Luscher et al., 1997) (Fig. 2). (3) All effects of BzATP could be reversed by the selective A1 receptor antagonist DPCPX (Lohse et al., 1987) (Figs. 1, 3, 8).
It should be noted, however, that the concentration of DPCPX used in our study (300 nM -1/H9262M) is not specific for A1 receptors but can also antagonize A2B receptors (Fredholm et al., 2001). In light of the previous studies and the very rapid onset of the DPCPX effect, however, A1 receptors appear to be the most likely candidates mediating the actions of BzATP.

Although these experiments suggest the involvement of A1 receptors, they do not identify the ligand that binds to the receptors. In contrast to the catabolism of ATP, dephosphorylation of BzATP should produce Bz-adenosine and not adenosine, because the benzoyl–benzoyl group is attached to C3 of the ribose and therefore will not be removed together with the phosphates (Fig. 9). On the other hand, for this modified ribose, Bz-adenosine is unlikely to be a potent A1 receptor agonist (van Galen et al., 1994; Klotz, 2000) or to be a substrate of adenosine deaminase, which degrades adenosine and is inhibited by many adenosine derivates (Cristalli et al., 2001). Despite that, BzATP and adenosine induced GIRK currents of very similar amplitude (Fig. 2), and this effect of BzATP could be completely reversed by coapplication of adenosine deaminase (Fig. 3). We hypothesized that after superfusion of BzATP, adenosine is released from intracellular compartments. Cellular release and uptake of adenosine in the brain are mediated by several types of membranous nucleoside transporters (Hyde et al., 2001; Lee et al., 2001; Baldwin et al., 2004). Recent work has shown that NTrans are expressed and functional in rodent hippocampus (Anderson et al., 1999; Sperlagh et al., 2003). Indeed, challenging NTrans with inosine (Yao et al., 1997; Li et al., 2001) reversed the outward current induced by BzATP (Fig. 4). It is important to note that in contrast to adenosine receptors and adenosine deaminase, nucleoside transporters have a rather broad range of substrate selectivity. They transport the full spectrum of nucleosides and even structurally weakly

Figure 8. Inhibition of mossy fEPSPs by BzATP (65 μM) is unchanged in P2X7−/− mice. A, Time course of fEPSP amplitude and example traces from time points are indicated by lowercase letters. DPCPX (1 μM) reverses the depression of fEPSPs by BzATP (65 μM). Horizontal bars represent the drug application time. Compare with Figure 1A, B. As in A but fEPSPs were recorded in the presence of 1 μM DPCPX. Note that BzATP does not depress fEPSPs under these conditions. B, Summary of the effects of BzATP on mossy fiber fEPSPs in the absence (n = 4) or presence (n = 3) of DPCPX (1 μM).

Figure 9. Hypothetical scheme to explain the A1 receptor-mediated action of ATP and BzATP in hippocampal slices. A, In three steps, ATP and BzATP are converted to adenosine and Bz-adenosine, respectively. The enzymes involved are E-NTPDases and SNTase and can be inhibited by ARL 67156, ITP, and concanavalin A. Adenosine deaminase (ADA) metabolizes adenosine. DPCPX competitively inhibits A1 receptor activation. Bz-adenosine cannot activate A1 receptors and cannot be further metabolized in the extracellular space because of lack of extracellular esterase activity (Satoh and Hosokawa, 1998). Instead, Bz-adenosine is transported intracellularly via NTrans, where it is converted to adenosine. In turn, intracellular accumulated adenosine is released in the extracellular space where it activates A1 receptors and is a substrate for ADA (Cristalli et al., 2001). Inosine and SB 203580 inhibit BzATP-induced activation of A1 receptors but do not affect the activity of either ATP or adenosine because they suppress only NTrans. Metabolites are placed in squares. Inhibitors and enzymes are printed in italic. Continuous arrows indicate conversion, transport, or positive modulation. Dashed arrows denote inhibition. ex, Extracellular; in, intracellular. B, Structure image of BzATP. Note the large benzoyl–benzoyl group, which makes it unlikely that Bz-adenosine is an A1 receptor ligand or an ADA substrate.
related antiviral drugs (Hyde et al., 2001). Based on the reversal of BzATP current by inosine, we suppose that NTrans mediate a hetero-exchange of Bz-adenosine and adenosine. Once BzATP has been transported intracellularly by NTrans, the benzoylbenzoyl group will removed by the required esterase activity, which is restricted to intracellular compartments (Satoh and Hosokawa, 1998). In turn, adenosine will be released in the extracellular space for equilibration (Fig. 9, scheme).

Additional evidence for the involvement of NTrans in the effects of BzATP stems from our experiments using the p38 MAP kinase inhibitor SB 203580, which was recently shown to potently inhibit NTrans in a culture system (Huang et al., 2002). SB 203580 blocks the inhibition of mossy fiber transmission by BzATP, and for this reason Armstrong et al. (2002) suggested that BzATP activates a MAP kinase-dependent pathway. Involvement of a MAP kinase pathway seems to be unlikely for two reasons. First, we could show that BzATP-induced depression of mossy fiber transmission is caused by activation of A1 receptors (Fig. 3), which inhibit presynaptic Ca2+ channels via G\text{GinA}-proteins (Wu and Saggau, 1997; Kamiya et al., 2002). Second, in our study, SB 203580 also reversed the A1-mediated activation of GIRK channels by BzATP, whereas it did not diminish the activation of GIRK channels by adenosine (Fig. 4). Thus, the function of the receptors and the associated downstream cascades (G\text{GinA}-proteins) (Lusch et al., 1997) are not affected by SB 203580. Rather it appears that SB 203580 hinders the formation of adenosine after perfusion of BzATP. Because SB 203580 specifically decreased the activation of GIRK currents by BzATP but not that caused by ATP and because SB 203580 was inactive in our in vitro assay, SB 203580 cannot be an inhibitor of ecto-nucleotidases. Alternatively, SB 203580 could be an inhibitor of NTrans in hippocampal slices as well. This would explain the differential depression of BzATP versus ATP effects (Fig. 4) and thus represents the most parsimonious explanation of the inhibitory effect of SB 203580.

ITP (2 mM) potently inhibited the activation of GIRK current by BzATP (20 μM) (Fig. 4), and we reasoned that this inhibitory action was attributable to competitive antagonism at the binding sites of E-NTPDases; however, ITP is a substrate of E-NTPDases well, and therefore it will be hydrolyzed to inosine. As above, inosine competes with Bz-adenosine for NTrans binding sites. Bath application of 2 mM ITP could lead to the formation of sufficient inosine to antagonize the binding of Bz-adenosine to NTrans. Thus, ITP may inhibit the action of BzATP by two means: competitive binding to E-NTPDase (ITP) and competitive binding to NTrans (inosine). In contrast, activation of GIRK channels by ATP does not require NTrans (see above). Therefore, the inhibition of ATP-induced currents by ITP should be caused solely by the decreased catabolism of ATP by E-NTPDases. Indeed, 2 mM ITP reduced the activation of GIRK channels by BzATP more strongly (completely) (Fig. 4) than the activation of GIRK channels by 30 μM ATP (to 51 ± 18%; n = 5; data not shown).

Taken together, we favor the following scenario of BzATP-mediated activation of A1 receptors (Fig. 9): ecto-nucleotidases catalyze BzATP to Bz-adenosine, which is transported intracellularly via NTrans. Then, intracellular esterases remove the Bz group, and the accumulating adenosine is released via nucleoside transporters and finally activates extracellular A1 receptors. Which particular type of NTrans is involved and whether it is placed on glial, neuronal or endothelial elements needs to be clarified in future studies.

The question remains whether there are functional P2X receptors on mossy fiber terminals. Strong P2X immunoreactivity of mossy fibers terminals has been reported (Armstrong et al., 2002; Sperlach et al., 2002), and we could reproduce this staining pattern in both mouse (Fig. 7B) and rat (data not shown) hippocampus using the same antibodies. Depression of mossy fiber synaptic transmission by BzATP, however, was not different between wild-type and P2X-\text{\text{\textsuperscript{7}}}−/− mice, suggesting that P2X function is not involved. On the other hand, it is possible that we did not observe a P2X-dependent effect because metabolism of BzATP precludes a sufficiently high agonist concentration within the slice. In fact, it was suggested that the catabolism of locally applied ATP is nearly quantitative (Dumwiddie et al., 1997). To exclude this possibility, we tested for functional P2X receptors, when ectonucleotidase activity was challenged by ITP (Fig. 6). A widely used and sensitive assay to assess the function of P2X receptors is to monitor how intracellular Ca2+ accumulates during opening of the Ca2+-permeable ionotropic P2X receptor (Schilling et al., 1999; Naemsch et al., 2001; James and Butt, 2002; North, 2002; Noble et al., 2003). We adopted this approach by loading mossy fiber terminals with a high-affinity Ca2+ indicator. Despite the use of long application times, BzATP did not elevate Ca2+ in mossy fiber terminals. This finding casts doubts on whether the antigen detected by the putative specific antibodies on mossy fiber terminals (Armstrong et al., 2002; Sperlach et al., 2002; this study) truly represents P2X7 protein. We tested the specificity of the antibodies in two different strains of P2X7 mice. In light of the negative results of the functional assay, it was not too surprising that the antibodies labeled the mossy fiber projection of both strains of mice in exactly the same manner as in wild-type mice (Fig. 7). Moreover, electron microscopy revealed that the detected antigen is not located at the presynaptic cytoplasmic membrane as would be expected for a transmitter gated receptor. Rather, the antigen is associated with intracellular presynaptic vesicles. We observed a similarly clear labeling of the molecular layer of the cerebellar cortex as well as of cerebellar glomeruli (unpublished observations); however, immunoreactivity in cerebellum was unchanged in knock-out mice as well. Thus, it seems that the antibodies cross-react at certain synapses with a presynaptic non-P2X7 protein.

Because the modulation of transmitter release was not different between wild-type and P2X7−/− mice, our data also indicate that there is no P2X7-mediated metabotropic (not Ca2+ mediated) action that interferes with synaptic transmission. Our findings are in accordance with the general understanding that this receptor subtype is expressed predominantly in immune cells (Collo et al., 1997), where it is responsible for the release of cytokines (Le Feuvre et al., 2002). Indeed, it seems hard to imagine for what reason synaptic terminals should carry P2X7 receptors that are able to form large cytolytic pores and would induce cell death after activation by the transmitter ATP (Brough et al., 2002; Le Feuvre et al., 2002). On the other hand, on the basis of our data, we cannot completely exclude that in addition to some P2X7-like antigen there are some “true” P2X7 receptors present on mossy fiber terminals that act exclusively on targets that are not involved in synaptic transmission; however, we consider this very unlikely, because in a former study (Kukley et al., 2001) we used another antibody directed against P2X7 and detected no labeling of the mossy fiber projection at all (our unpublished observation). Because all studies that reported presynaptic P2X7 used the same antibody (raised against residues 576–595 of rat P2X7; Alomone Labs, Chemicon, Temecula, CA), it will be interesting to see whether future studies can confirm the presence of presynaptic P2X7 at other nerve terminals (Deuchars et al., 2001; Miras-
Portugal et al., 2003; Atkinson et al., 2004; Puthussery and Fletcher, 2004).

In conclusion, the present study demonstrates that the supposedly stable P2X7 agonist BzATP is enzymatically converted by hippocampal slices before it leads to release of adenosine and activation of adenosine receptors. No evidence could be obtained for a functional role of P2X7 receptors on mossy fiber terminals. Rather, all effects that were observed after application of BzATP could be explained by the activation of adenosine A1 receptors.

References

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