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Supersensitivity of $P2X_7$ receptors in cerebrocortical cell cultures after *in vitro* ischemia

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Abstract

Neuronally enriched primary cerebrocortical cultures were exposed to glucose-free medium saturated with argon (*in vitro* ischemia) instead of oxygen (normoxia). Ischemia did not alter $P2X_7$ receptor mRNA, although serum deprivation clearly increased it. Accordingly, $P2X_7$ receptor immunoreactivity (IR) of microtubuline-associated protein 2 (MAP2)-IR neurons or of glial fibrillary acidic protein (GFAP)-IR astrocytes was not affected; serum deprivation augmented the $P2X_7$ receptor IR only in the astrocytic, but not the neuronal cell population. However, ischemia markedly increased the ATP- and 2′-3′-*O*-(4-benzoylbenzoyl)-adenosine 5′-triphosphate (BzATP)-induced release of previously incorporated [^{3}H]GABA. Both Brilliant Blue G and oxidized ATP inhibited the release of [^{3}H]GABA caused by ATP application; the Brilliant Blue G-sensitive, $P2X_7$ receptor-mediated fraction, was much larger after ischemia than after normoxia. Whereas ischemic stimulation failed to alter the amplitude of ATP- and BzATP-induced small inward currents recorded from a subset of non-pyramidal neurons, BzATP caused a more pronounced increase in the frequency of miniature inhibitory postsynaptic currents (mIPSCs) after ischemia than after normoxia. Brilliant Blue G almost abolished the effect of BzATP in normoxic neurons. Since neither the amplitude of mIPSCs nor that of the muscimol-induced inward currents was affected by BzATP, it is assumed that BzATP acts at presynaptic $P2X_7$ receptors. Finally, $P2X_7$ receptors did not enhance the intracellular free Ca^{2+} concentration either in proximal dendrites or in astrocytes, irrespective of the normoxic or ischemic preincubation conditions. Hence, facilitatory $P2X_7$ receptors may be situated at the axon terminals of GABAergic non-pyramidal neurons. When compared with normoxia, ischemia appears to markedly increase $P2X_7$ receptor-mediated GABA release, which may limit the severity of the ischemic damage. At the same time we did not find an accompanying enhancement of $P2X_7$ mRNA or protein expression, suggesting that receptors may become hypersensitive because of an increased efficiency of their transduction pathways.

Keywords: ATP, cortical cell culture, ischemia, $P2X_7$ receptor, receptor up-regulation.

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High concentrations of ATP have been reported to activate a certain subtype of the ionotropic P2X receptor ($P2X_7$; Surprenant *et al*. 1996; Khakh *et al*. 2001). This receptor

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Abbreviations used: ACSF, artificial cerebrospinal fluid; AP-5, D(–)-amino-5-phosphonopentanoic acid; ATP, adenosine 5′-triphosphate; BzATP, 2′-3′-*O*-(4-benzoylbenzoyl)-adenosine 5′-triphosphate; CNQX, 6-cyano-7-nitroquinoxaline-2,3-dione; CSC, 8-(3-chlorostyryl)caffeine; C_T values, cycle-threshold values; DIV, days *in vitro*; DPCPX, 8-cyclopentyl-1,3-dipropylxanthine; GFAP, glial fibrillary acidic protein; IR, immunoreactivity; MAP2, microtubule-associated protein 2; MCAO, middle cerebral artery occlusion; mIPSC, miniature inhibitory postsynaptic current; MRS 2179, 2′-deoxy-N^6-methyladenosine 3′,5′-bisphosphate; NMDA, *N*-methyl-D-aspartate; oxiATP, oxidized ATP; PPADS, pyridoxal-phosphate-6-azophenyl-2′,4′-disulfonic acid.

allows the passage of small cations on immediate activation by agonists; long-lasting activation, however, leads to a progressive dilation of the ion channel and formation of a large pore (permeable to organic molecules and dyes), and membrane blebbing (Virginio *et al*. 1999; North 2002).

The $P2X_7$ receptor is thought to be expressed predominantly on antigen-presenting immune cells and epithelia (Rassendren *et al*. 1997). Astrocytes and Müller cells of the retina have also been described to bear $P2X_7$ receptors, which possibly mediate inflammation and subsequent proliferation (Pannicke *et al*. 2000; Gendron *et al*. 2003). In addition, these astrocytic receptors release glutamate (Duan *et al*. 2003) and GABA (Pannicke *et al*. 2000) participating in astrocyte–neuron communication. More recently, $P2X_7$ receptors were identified on neurons of the central and peripheral nervous system by single cell RT–PCR, immunohistochemistry and functional investigations (Deuchars *et al*. 2001; Hu *et al*. 2001; Sperlágh *et al*. 2002; Miras-Portugal *et al*. 2003; Allgaier *et al*. 2004; Wang *et al*. 2004). Unfortunately, the value of antibodies in identifying this neuronal target were questioned by the demonstration of a pseudo-immunoreactivity (IR) for $P2X_7$ receptors in the hippocampus of $P2X_7^{-/-}$ mice (Sim *et al*. 2004; Kukley *et al*. 2004), although the $P2X_7$ receptor-mediated transmitter release disappeared in the knockout animals (Papp *et al*. 2004).

In support of the assumption that cerebral ischemia aggravates brain injury via the efflux of ATP, both intrastriatally injected ATP (Ryu *et al*. 2002) and middle cerebral artery occlusion (MCAO; Kharlamov *et al*. 2002) caused lesions, which were prevented by the application of the wide-range P2 receptor antagonists suramin and pyridoxalphosphate-6-azophenyl-2′,4′-disulfonic acid (PPADS). Mechanical (Franke *et al*. 2001) or ischemic injury (Collo *et al*. 1997; Franke *et al*. 2004) has been shown to lead to the expression of previously absent $P2X_7$ receptor IR on microglia, astrocytes and neurons. Further, a large number of investigations in cell culture preparations strongly suggest that P2 receptors are involved in ischemic neuronal damage (Volonte *et al*. 1999; Cavaliere *et al*. 2001). More specifically, oxygen/glucose deprivation was shown to up-regulate $P2X_7$ receptor IR in primary cultures of cerebellar granule neurons (Cavaliere *et al*. 2002) and in CA1 pyramidal neurons of organotypic hippocampal cultures (Cavaliere *et al*. 2004). It was hypothesized that after ischemia, the excessive release of the excitotoxic ATP up-regulates $P2X_7$ receptors and thereby facilitates the cellular necrosis caused by metabolic limitation.

The aim of the present study was to investigate in neuronally enriched primary cortical cultures, whether an ischemic stimulus of relatively short duration increases $P2X_7$ receptor-mediated functions, and whether such an effect correlates with a corresponding increase in $P2X_7$ mRNA and IR. We found that ischemia markedly increases the $P2X_7$ receptor-mediated release of GABA without a major change in the number of these receptors. It is concluded that $P2X_7$ receptors situated at the terminals of GABAergic neurons may be responsible for a limitation of the ischemic neuronal damage in the cell culture system.

Materials and methods

Preparation of cortical cell cultures

Cell cultures were prepared from rat fetuses at gestational day 16 and grown as described earlier (Günther *et al*. 2002; Reinhardt *et al*. 2003). In short, a cell suspension made with a 1:1 mixture of Dulbecco's modified Eagle's medium and Nutrient F12 supplemented with 20% fetal calf serum, 2.2 mM L-glutamine, 15 mM HEPES, 50 μg/mL gentamicin, and 30 mM D-glucose was seeded into poly-L-lysine-coated polystyrol dishes or on glass coverslips at a density of 5×10^5 cells per dish/coverslip and cultured at 37°C in a humidified atmosphere containing 5% CO_2/95% air. After 5 days of cultivation, the medium was replaced by Neurobasal™ medium supplemented with 0.5 mM glutamine, serum-free supplement B27 (v/v 50/1) and 50 μg/mL gentamicin. At 5 to 10 days later the neurobasal medium was removed and the cell cultures were rinsed twice with artificial cerebrospinal fluid (ACSF) (composition in mM: NaCl 140; KCl 5; $MgCl_2$ 2, $CaCl_2$ 2; HEPES 10; glucose 11; pH 7.4 adjusted with NaOH). Cultures were used thereby on days 10–15 (10–15 DIV) for most experiments. Exceptions were fura-2 measurements, where culturing until day 5 and from day 7 onward was identical to that described earlier, but in between cytosine β-arabino-furanoside (10 μM) was added for 24 h.

Release experiments

The [^{3}H]GABA release experiments were carried out with slight modifications of previous experimental protocols (Sperlágh *et al*. 2002). The neurobasal medium in each culture dish was replaced with 1 mL HEPES buffer of the following composition (in mM): NaCl 135; KCl 5; $MgSO_4$ 0.6; $CaCl_2$ 1; glucose 6; HEPES 10; pH 7.3; saturated with O_2. When the experiment was performed under normal conditions, the culture dishes were kept under O_2-saturated atmosphere for 1 h, then the buffer was replaced with 1 mL of the same buffer containing 0.5 μCi 4-amino-*n*-[2,3-^{3}H]butyric acid ([^{3}H]GABA, specific activity 86.0 Ci/mmol; Amersham Pharmacia, Braunschweig, Germany) and β-alanine (1 mM) for 20 min. β-Alanine has been described to prevent tritium uptake into glial cells but not neurons (Iversen and Kelly 1975).

After incubation with [^{3}H]GABA, the cells were covered with an 80 μm-pore nylon mesh for physical protection from the superfusion, and were then subjected to a subsequent 15-min washout with HEPES buffer at a rate of 2 mL/min. Upon termination of the washout, 1-min samples were collected from the effluent. When experiments were performed under ischemic conditions, the neurobasal medium was replaced with glucose-supplemented and O_2-saturated HEPES buffer, and the dishes were kept under O_2-saturated atmosphere as described above, but only for 30 min. Then, glucose was substituted with equimolar saccharose, and O_2 was replaced with argon in the atmosphere and in the solutions. From this time onward, all solutions were glucose-free and argon-saturated. Thus, the second 30 min of pre-incubation, the 20 min

of incubation with the isotope and the 15 min of washout yielded 65 min of ischemic pre-treatment before the sample collection. All experiments were carried out at room temperature. To minimize the formation of GABA metabolites, all solutions contained aminooxyacetic acid (100 μM) in normal and ischemic conditions as well. This 65-min ischemic stimulus caused a somewhat larger decrease of the ATP/ADP ratio from 6.1 ± 0.9 ($n = 5$) to 0.39 ± 0.01 ($n = 6$; $p < 0.05$; by ~90%) than a 30-min ischemic stimulus (~60% depression of the ATP/ADP ratio) used for immunocytochemistry, electrophysiology and Ca^{2+} microfluorimetry (see below).

During the sample collection period, two kinds of protocol were applied as described previously (Sperlágh *et al*. 2002). According to the first protocol, the normoxic and ischemic cell cultures were challenged with increasing concentrations of ATP or BzATP in each experiment, for 1 min every 5–6 min. Of the $P2X_7$ receptor antagonists, Brilliant Blue G (1 μM) and PPADS (30 μM) were present in all solutions used, from the beginning of pre-incubation. Oxidized ATP (oxiATP; 300 μM) was present only during the 95-min incubation period; however, this time was sufficient for the antagonist to irreversibly inhibit $P2X_7$ receptors.

Concentration–response curves were fitted using the following logistic function (SigmaPlot, SPSS, Erkrath, Germany),

$$E = E_{\min} + (E_{\max} - E_{\min})[1 + (EC_{50} + A)^n],$$

where E is the steady-state effect produced, A is the agonist concentration, $E_{\max}$ and $E_{\min}$ are the maximal and minimal effects, respectively, n is the Hill coefficient, and EC_{50} is the concentration of agonist producing 50% of $E_{\max}$.

According to the second protocol, with 10-min delay, the ischemic cell cultures were stimulated with ATP (3 mM) twice (S_1 and S_2). Six minutes before S_2, various drugs, i.e. tetrodotoxin (1 μM), D(–)-amino-5-phosphonopentanoic acid (AP-5, 50 μM) plus 6-cyano-7-nitroquinoxaline-2,3-dione (CNQX, 10 μM), Reactive Blue 2 (10 μM) plus 2′-deoxy-N^6-methyladenosine 3′,5′-bisphosphate (MRS 2179, 10 μM), and 8-cyclopentyl-1,3-dipropylxanthine (DPCPX, 0.25 μM) plus 8-(3-chlorostyryl)caffeine (10 μM) were applied. The latter two compounds were dissolved in ethanol, whereas all the other drugs were dissolved in water. The final concentration of ethanol (0.25%) had no effect on the [^{3}H]GABA release.

The release of [^{3}H]GABA was calculated as percentage of the amount of radioactivity in the neurons at the sample collection time (fractional release; FR%) (Sperlágh *et al*. 2002). It was found that [^{3}H]GABA represents the majority (~95%) of total tritium efflux.

Incubation conditions for electrophysiology, Ca^{2+} microfluorimetry, immunocytochemistry, and real-time polymerase chain reaction

A culture dish was rapidly transferred to 500 mL of ACSF (see above) and incubated for 90 min at 37°C. The control solution was saturated with 100% oxygen (pO_2, 710–740 mmHg; pH, 7.3). The oxygen saturation was controlled by a Clark type electrode via an oxymeter (StrathKelvin instruments, Glasgow, Scotland). A condition termed '*in vitro* ischemia' was achieved by placing the dishes into glucose-free ACSF separated from the environmental air in a desiccator and carefully gassed with 100% argon (pO_2, 1–2 mmHg) for at least 30 min before starting the incubation period. A glucose-free medium was prepared by omitting 11 mM glucose from the standard ACSF and by increasing the content of NaCl to 151 mM.

Incubation was performed according to two different time-schedules: The first 60-min incubation was always in normoxic and glucose-containing medium followed by a 30 (or in some experiments 60)-min incubation period with either normoxic and glucose-containing medium (normoxia) or hypoxic and glucose-free medium (ischemia). Immediately after the total incubation period of 90 (or 120) min, the cells were incubated with normal ACSF solution before and during electrophysiological recording or loading with fura-2 and subsequent microfluorimetry. In some of the experiments, before electrophysiology and fura-2 microfluorimetry, there was no 90-min pre-incubation in a desiccator (normal condition). It has previously been shown that a 30-min *in vitro* ischemia (hypoxia and glucose-deficiency) markedly depressed the ATP/ADP ratio (by ~60%) in comparison with normoxic controls (Günther *et al*. 2002; Reinhardt *et al*. 2003).

Whole-cell patch-clamp recordings

Membrane currents were recorded in the whole-cell configuration of the patch-clamp method at room temperature (20–22°C) using an Axopatch 200 B amplifier (Axon Instruments, Union City, CA, USA) (Allgaier *et al*. 2004). The pipette solution contained (in mM): CsCl 140, $MgCl_2$ 1, $CaCl_2$ 1, HEPES 10, EGTA 10, MgATP 1.5, LiGTP 0.3, *N*-methyl-lidocaine iodide (QX-314) 2; pH adjusted to 7.3 with CsOH. The holding potential was −60 mV. For current-clamp measurements, CsCl was replaced in the pipette solution by equimolar KCl and the pH was adjusted with KOH instead of CsOH. Currents were filtered at 2–5 kHz with the inbuilt lowpass-filter of the patch-clamp amplifier. Data acquisition and analysis were performed computer-controlled using pClamp 8.0 software (Axon Instruments; sampling rate, 3–10 kHz). An ACSF of known composition (see above) was used as the external recording solution. $GABA_A$ receptor-mediated miniature inhibitory postsynaptic potentials (mIPSCs) were measured at −60 mV, in the presence of AP-5 (50 μM), CNQX (10 μM) and tetrodotoxin (0.5 μM) in the external medium. mIPSCs were analysed using commercially available software (MiniAnalysis 4.3; Synaptosoft, Decatur, GA, USA).

ATP (1–10 mM), 2′-3′-*O*-(4-benzoylbenzoyl)-adenosine 5′-triphosphate (BzATP; 30–300 μM), Brilliant Blue G (0.3 μM) and muscimol (10 μM) were dissolved in ACSF; the pH of the agonist solutions in this and all subsequent experiments was balanced to 7.3. When we measured effects on the holding current, ATP and BzATP were applied by a pressurized fast-flow superfusion system (DAD-12; Adams and List, Westbury, NY, USA) for 1 s each, separated by drug-free intervals of 3 min. In experiments with muscimol, the $GABA_A$ agonist was applied for 2 s every 3 min; BzATP was superfused for 6 min, immediately after finishing the second application of muscimol. The same superfusion system was used to apply BzATP and Brilliant Blue G, when effects on the mIPSC amplitude and frequency were measured. Mean mIPSC amplitudes and frequencies were calculated during a control period of 3 min and during the last 3 min of the subsequent application of BzATP for 5 min. In separate experiments, BzATP, Brilliant Blue G, or BzATP plus Brilliant Blue G was applied for two subsequent 5-min periods each. The evaluation times lasted again for 3 min as mentioned above. The mean amplitude and frequency of mIPSCs were normalized with respect to the control values recorded during

the first 3-min period in drug-free ACSF. The changes were expressed as percentage potentiation of the time-matching controls recorded in drug-free ACSF for 15 min in total. Amplitude histograms were binned in 2-pA intervals.

Fura-2 microfluorimetry

Cell cultures, after the 30-min incubation procedure in normoxic or ischemic ACSF (see above), were washed in superfusion medium (composition in mM: NaCl 133; KCl 4.8; KH_2PO_4 1.2; $CaCl_2$ 1.3; HEPES 10; glucose 10; pH 7.4; room temperature) and loaded in this solution with fura-2 acetoxymethyl ester (5 μM, for 30 min). After loading, all cultures were washed again to remove extracellular traces of the dye. The coverslips were then placed on the stage of an inverted microscope with epifluorescence optics (Diaphot 200; Nikon, Düsseldorf, Germany). Throughout the experiments, the cells were continuously superfused at 0.8 mL/min by means of a roller pump. A high external K^+-containing medium (50 mM, Na^+ replaced by an equimolar amount of K^+; 3 s superfusion), and ATP or BzATP (300 μM each; 10 s superfusion) were applied directly to single cells by pressure every 12 min, using a fast-flow superfusion system (DAD12, Adams and List). Cyclopiazonic acid (10 μM), a Ca^{2+}-free solution (with 1 mM EGTA), Brilliant Blue G (1 μM) or PPADS (30 μM) were superfused 10 min before and during the next pressure application of ATP or BzATP.

Fluorescence ratio measurements were made on single, morphologically identified multipolar non-pyramidal neurones (over the cell somata or proximal dendrites) and astrocytes with a dual wavelength spectrometer (alternating excitation at 340/380 nm). Fura-2 fluorescence was measured at 510/520 nm by a microscope photometer attached to a photomultiplier detection system (Ratiomaster System; PTI, Wedel, Germany). The agonist-induced rise of $[Ca^{2+}]_i$ was defined as the peak increase in the Δ fluorescence ratio (i.e. the fluorescence ratio 340/380 nm in response to the agonist minus the basal fluorescence ratio). Data acquisition and analysis were performed computer-controlled by using commercially available software (FeliX, Version 1.1; PTI). Calibration of $[Ca^{2+}]_i$ was performed by determining Ca^{2+}-saturated fura-2 signals (R_{max}) in the presence of 10 μM ionomycin (Mg^{2+}-free buffer), and Ca^{2+}-free signals (R_{min}) in the presence of 25 mM EGTA (Ca^{2+}-free buffer), respectively (Fischer *et al.* 2002). Pressure application of K^+ (50 mM for 3 s) evoked a Δ fluorescence ratio of 2.76 ± 0.26, which corresponds to a mean maximum elevation of $[Ca^{2+}]_i$ from basal 75.1 ± 4.1 nM to 845.4 ± 93.4 nM ($n = 12$ cells).

RNA isolation and real-time polymerase chain reaction using SYBR-Green chemistry

Total RNA was prepared from 5×10^5 cultured cells using the RNeasy Mini Kit (Qiagen, Hilden, Germany) according to the manufacturer's directions. The amount of RNA was quantified by measuring the absorbance at 260 nm and 280 nm in a spectrophotometer (Eppendorf, Wesseling, Germany). The quality of the extracted RNA was visualized on a 1% agarose gel after electrophoresis.

A two-step reverse transcription was performed by using SuperScript II (Life Technologies, Eggenstein, Germany). cDNA was purified with QIAquick PCR Purification Kit (Qiagen). The SYBR Green real-time PCR assay was carried out in 20 μL PCR mixture volume consisting of 10 μL of 2 × QuantiTect SYBR Green PCR Master Mix (Qiagen, containing HotStar Taq DNA polymerase, PCR-Buffer, dNTP mix and SYBR Green I), 2 μL reverse and forward primers each at 0.5 μM, 2-μL samples of cDNA and 4 μL RNase-free water. The reactions comprised 15 min of initial denaturation at 95°C, 45 cycles of denaturation (94°C for 30 s), annealing (40°C for 20 s), and extension (72°C for 10 s). All reactions were made in duplicate with no template control. Amplification, data acquisition and analyses were carried out by Light Cycler instrument (Roche Diagnostics, Mannheim, Germany) using Light Cycler 5.3.2 software (Roche). The identity of the PCR products was confirmed by sequencing. The sequence of $P2X_7$ primers were as follows: forward 5′-TGTCCCTATCTCTCCACG-ACTCAC-3′ and reverse 5′-ATTTCCACACTGGCACCAACTC-GG-3′ (GenBank accession number NM011027). The amplification with this pair of oligonucleotides yielded a 119 bp fragment.

To determine the suitable endogenous reference gene, we examined cycle-threshold (C_T) values of several endogenous reference genes, such as 18S rRNA, ribosomal protein L28, and β-actin. The results indicate that 18S rRNA expression offers superior consistency during normoxic and ischemic conditions. The primer sequences for the housekeeping gene 18S rRNA were as follows: forward 5′-TCAAGAACGAAAGTCGGAGGTT-3′ and reverse 5′-GGTCATCTAAGGGCATCACAG-3′. The C_T values for $P2X_7$ were normalized with respect to the C_T values for 18S rRNA.

Immunofluorescence and confocal microscopy

$P2X_7$ receptor IR in cultured cortical neurons was detected with polyclonal rabbit anti-$P2X_7$ antibodies raised against highly purified peptides corresponding to amino acids 576–595 of the carboxyl terminus of the cloned rat $P2X_7$ receptor. The cultures were washed twice for 5 min in Hank's buffered saline solution and fixed in ice-cold methanol. For permeabilization and blocking, the cells were pre-treated with 0.1% Triton X-100 and 5% fetal calf serum in Tris-buffered saline (0.05 M, pH 7.6) for 30 min. Then, the cultures were incubated with an antibody mixture of the mouse microtubule-associated protein 2 (MAP2, 1:1000) and/or mouse glial fibrillary acidic protein (GFAP, 1:1000) and the rabbit $P2X_7$ (1:600) receptor antibody, in combination with Cy2-conjugated goat anti-mouse IgG (1:400), Cy3-conjugated goat anti-rabbit IgG (1:1000), and Cy5-conjugated streptavidin (1:800). For the GABA-immunnofluorescence study, the cultures were fixed with paraformaldehyde (4%)/glutaraldehyde (0.25%) for 10 min at 4°C followed by washing and blocking as described above. The cell cultures were incubated with the antibody mixture of rabbit anti-$P2X_7$ and mouse anti-GABA (1:100) and the respective Cy2/Cy3-labeled secondary antibodies. After intensive washing and mounting on slide glasses, all stained sections were dehydrated in a series of graded ethanol, processed through *n*-butylacetate and covered with entellan (Merck, Darmstadt, Germany). Control experiments were carried out without the primary $P2X_7$ receptor antibody or by pre-adsorption of the antibody with the immunizing peptides. The immunofluorescence was investigated by a confocal laser scanning microscope (LSM 510, Zeiss, Oberkochen, Germany) at an excitation wavelength of 633 nm (helium/neon2, blue Cy5-immunofluorescence), 543 nm (helium/neon1, red Cy3-immunofluorescence), and 488 nm (argon, yellow-green Cy2-immunofluorescence).

For statistical evaluation, 50 GABA-, MAP2- or GFAP-immunoreactive cells were counted in each culture dish after each treatment schedule (untreated, normoxia, ischemia). Then cells with double IR for $P2X_7$ plus GABA, MAP2 or GFAP were also counted and expressed as a percentage of the total cell population of 50.

Materials and drugs

The following antibodies and conjugated markers were used: mouse monoclonal anti-GABA (Clone GB-69; Sigma-Aldrich, Taufkirchen, Germany), rabbit anti-$P2X_7$ receptor subtype (intracellular C-terminus binding, Lot #: AN-03; Alomone Laboratories, Jerusalem, Israel); mouse anti-MAP2 (Chemikon, Temecula, CA, USA); Cy2-conjugated goat anti-mouse IgG, Cy3-conjugated goat anti-rabbit IgG (Jackson Immuno Research, Baltimore, USA).

The following drugs were used: adenosine 5′-triphosphate disodium salt (ATP), AP-5, aminooxyacetic acid, BzATP, Coomassie Brillant Blue G, 8-(3-chlorostyryl)caffeine (CSC), CNQX, DPCPX, cyclopiazonic acid, EGTA, MRS 2179, cytosine β-D-arabino-furanoside, fura-2 acetoxymethyl ester (Fura-2/AM), muscimol, L-glutamine, N-methyl-D-aspartate (NMDA), oxiATP, PPADS, Reactive Blue 2, tetrodotoxin (all from Sigma-Aldrich, Taufkirchen, Germany); bafilomycin, *N*-methyl-lidocaine iodide (QX-314; Tocris, Bristol, U.K); 4-amino-*n*-[2,3-^{3}H]butyric acid ([^{3}H]GABA, specific activity, 86.0 Ci/mmol; Amersham Pharmacia Biotech, Buckinghamshire, UK); Dulbecco's modified Eagle's medium, Dulbecco's medium Nutrient F12, Neurobasal medium, gentamycine, trypsine (Life Technologies, Karlsruhe, Germany); fetal calf serum (Seromed, Berlin, Germany). All chemicals were of analytical grade.

Statistics

Means ± SEM of *n* determinations are shown. Multiple comparisons with the control value were made by one-way analysis of variance (ANOVA) followed by the Kruskal–Wallis analysis and the Bonferroni's *t*-test. Two values were compared with the Dunnett's test or the Student's *t*-test as appropriate. Cumulative probability plots of mIPSCs were constructed for amplitudes and interevent intervals and compared using the Kolmogorov–Smirnov test. A probability level of 0.05 was considered as the limit of significance.

Results

Effect of *in vitro* ischemia on $P2X_7$ receptor mRNA and immunoreactivity in cortical neurons and astrocytes kept in culture

First, we investigated whether the $P2X_7$ receptor mRNA in cortical cell cultures is up-regulated by pre-incubation in ischemic, glucose-free ACSF saturated with argon, in comparison with pre-incubation in normoxic, glucose-containing ACSF (Fig. 1a). Control measurements were made in the normal serum-containing culturing medium. Both normoxic and ischemic ACSF time-dependently decreased the normalized cycle-threshold values indicating an increase of $P2X_7$ receptor mRNA in real-time PCR determinations. We assume that serum deprivation, which is a well-known apoptotic stimulus, enhances the transcription of $P2X_7$ receptors already within a period of 1–2 h. However, ischemia failed to further increase the $P2X_7$ mRNA over the normoxic values during the same time periods.

Next, we utilized immunocytochemistry and confocal laser scanning microscopy to characterize the prominent cell types in the cortical cultures investigated. Previous studies revealed that approximately 80–90% of neurons were GABA-immunoreactive (Fischer *et al*. 2002). In fact, many untreated neurons were labeled for GABA IR and a considerable population was co-labeled for GABA- and $P2X_7$ receptor IR (Fig. 1b, i, ii and iv). An antibody raised against a C-terminal epitope of the $P2X_7$ receptor was used in all experiments. Three different types of neurons (pyramidal, multipolar and bipolar) were morphologically identified. Especially the relatively small bipolar cell bodies co-expressed the GABA- and the $P2X_7$ IR with a high incidence (Fig. 1b, ii); larger multipolar neurons less often exhibited co-expression, whereas the assumedly glutamatergic pyramidal neurons usually did not stain for GABA (Fig. 1b, i). These findings are in partial contradiction to the finding that in the rat CNS the majority of the $P2X_7$ IR was localized at non-GABAergic neurons (Deuchars *et al*. 2001; Sperlágh *et al*. 2002; Atkinson *et al*. 2004). A possible reason for this discrepancy is that tissue damage during the culturing procedure may markedly up-regulate $P2X_7$ receptor expression in comparison with the *in vivo* conditions.

Of course a co-localization of the $P2X_7$- and GABA IR is not sufficient to decide whether the $P2X_7$ IR is situated on neurons or astrocytes, because both cell-types may synthesize GABA. To find out which cells express the $P2X_7$ IR, cortical cultures were labeled with the neuronal marker MAP2 and the astrocytic marker GFAP (Fig. 1b, iii). The $P2X_7$ receptor IR was co-localized both with MAP2 and GFAP IR on neurons and astrocytes, respectively. A statistical evaluation of these data revealed that whereas a high percentage of untreated GABA- and MAP2-immunopositive cells co-stained also for $P2X_7$ IR, only a low percentage of untreated GFAP-immunopositive cells exhibited also $P2X_7$ IR (Fig. 1b, iv). Further, both normoxic and ischemic pre-incubation for 30 min failed to alter the number of cells that co-expressed $P2X_7$ IR with GABA or MAP2 IR. In contrast, the number of cells co-expressing $P2X_7$ and GFAP immunopositivity was similarly enhanced both after normoxic and ischemic pre-treatment (Fig. 1b, iv). It is suggested that, in GFAP-immunopositive astrocytes, serum deprivation increased $P2X_7$ mRNA (see above) and its transcription to the corresponding receptor protein, within a period of 1–2 h. However, ischemia failed to further enhance the $P2X_7$ mRNA over the normoxic values during the same time periods.

When cell cultures were incubated with Tris-buffered saline instead of the primary antibody, or with primary antibody-serum that had been pre-absorbed with peptide

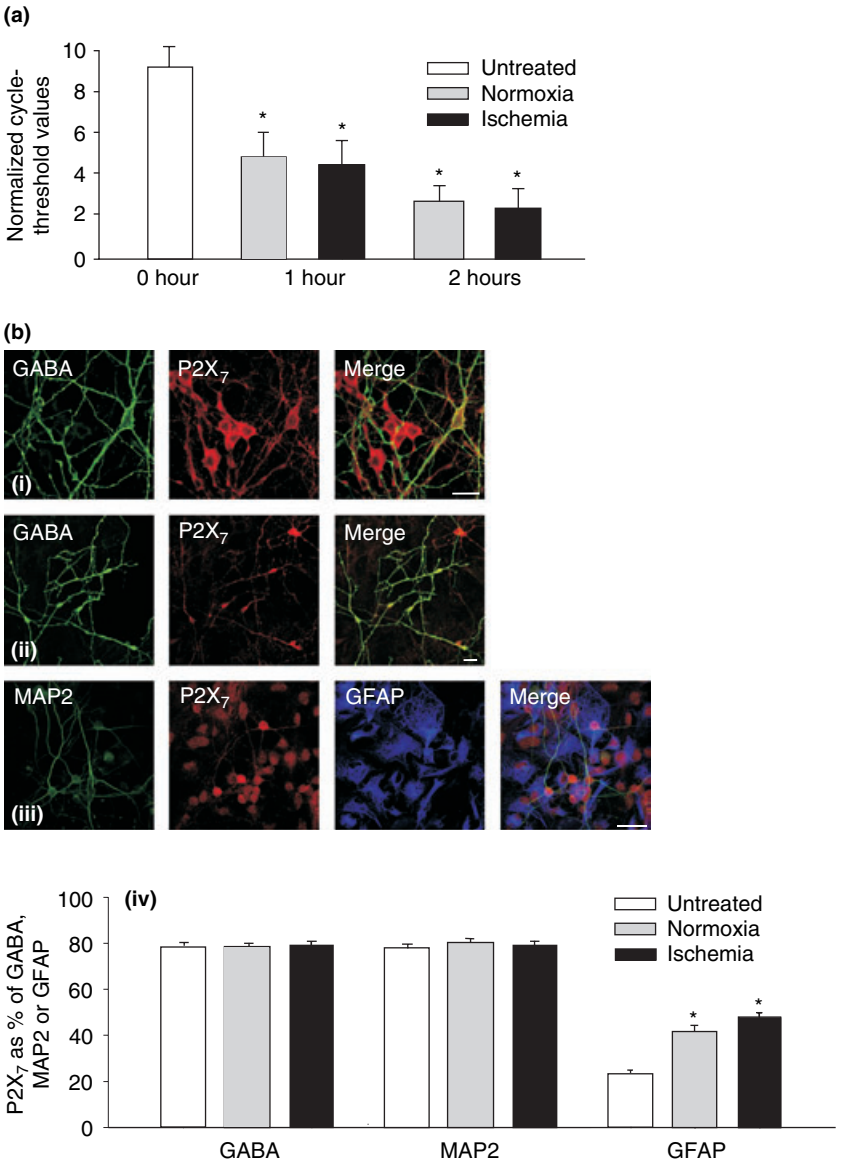

Fig. 1 Transcription and translation of $P2X_7$ receptors in cortical cell cultures of rats. (a) Increased synthesis of $P2X_7$ receptor mRNA by serum deprivation but not by ischemic pre-incubation for 1 or 2 h. The cycle-threshold (C_T) values normalized with respect to the endogenous reference gene 18S rRNA, were used as a measure of mRNA levels. Note that the transcription of the $P2X_7$ receptor is time-dependently increased by a replacement of the culturing medium by ACSF, irrespective of its glucose-content or its saturation by oxygen or argon. Untreated (culturing medium; empty columns), normoxic (ACSF plus oxygen; grey columns) and ischemic (glucose-free ACSF plus argon; black columns) cells both in (a) and (b). Means ± SEM of 7–15 experiments. *$p < 0.05$; statistically significant differences from untreated controls. (b) Immunocytochemical characterization of the prominent cell-types present in untreated cultures and co-localization of $P2X_7$ receptor IR with GABA, microtubule-associated protein 2 (MAP2) and glial fibrillary acidic protein (GFAP) IR. Confocal laser scanning microphotographs. (b, i) Co-localization of GABA (Cy2) and $P2X_7$ (CY3) IR at the cell body of one multipolar cell. (b, ii) Co-localization of GABA- and $P2X_7$ IR at a number of bipolar cells. Many cellular processes co-express GABA- and $P2X_7$ IR both in (b, i) and (b, ii). Scale bars in (b, i) and (b, ii), 20 μm. (b, iii) Co-localization of MAP2 (Cy2), $P2X_7$ (Cy3), and GFAP (Cy5) IR on neurons and astrocytes, as well as on cell bodies and processes/fibers. MAP2 and GFAP were used as neuronal and astrocytic markers, respectively. Scale bar, 50 μm. (b, iv) Cells with double-immunoreactivity for $P2X_7$ plus GABA, MAP2 or GFAP expressed as a percentage of the total cell population of 50 counted cells. Normoxic and ischemic pre-incubation was applied for 30 min. Means ± SEM of 50 cells from five culture dishes each. *$p < 0.05$; statistically significant difference from untreated control cells in the respective set of columns.

antigen for 1 h before use (1 μg of peptide per 1 μg of antibody), no immunofluorescence with either of the control procedures was observed.

ATP- as well as 2′-3′-*O*-(4-benzoylbenzoyl)-ATP induced stimulation of [^{3}H]GABA release from cortical cell cultures and interaction with Brilliant Blue G, oxidized ATP and pyridoxal-phosphate-6-azophenyl-2′, 4′-disulfonic acid

As previous studies revealed that approximately 80–90% of neurons in cerebrocortical cultures were GABA-immunoreactive (Fischer *et al*. 2002), storage pools for GABA were labeled by pre-incubation with [^{3}H]GABA (see Methods). After 15 min of washout, the spontaneous tritium efflux in the first 1-min sample was 0.180 ± 0.005% ($n = 11$) of the total tritium content in the cell culture. This value remained fairly constant until the end of the experiment. Subsequent to stimulation with 1-min superfusions of ATP (0.03–10 mM), a concentration-dependent elevation in the basal [^{3}H]GABA outflow was observed with an EC_{50} value of 340 μM (Fig. 2a, i and ii). The action of ATP was reversible upon washout (Fig. 2a, i). At 1 mM, the effect of ATP reached its plateau, and no further increase of the evoked [^{3}H]GABA release was observed for 3, 6, and 10 mM of ATP (Fig. 2a, i and ii). The relatively selective $P2X_7$ receptor agonist BzATP (0.1–1 mM; Ralevic and Burnstock, 1998), had no effect on the [^{3}H]GABA release (Fig. 2a, ii).

After ischemic pre-treatment, the basal [^{3}H]GABA release in the first 1-min sample was 0.188 ± 0.008% ($n = 13$, $p > 0.05$), and thereby not significantly different from that measured in a normoxic ACSF. ATP (0.3–30 mM) showed a lower potency but increased efficacy to release [^{3}H]GABA under these conditions (Fig. 2a, i and ii). The smallest ATP concentration that evoked reproducible [^{3}H]GABA release (0.6 mM) was ∼10-times higher than under normoxic conditions. The effect of ATP reached its maximum at 10 mM with an EC_{50} value of 4.3 mM (Fig. 2a, ii). The maximum response to ATP in an ischemic medium was ∼two-times higher than under normoxic condition.

As ATP had a lower potency in ischemia than in normoxia, it is possible that the ability of the cultured cells to release [^{3}H]GABA under energy deprivation is diminished. In an attempt to compare the release ability of these cells under normoxic and ischemic conditions, culture dishes were depolarized with a 25 mM K^+-buffer (equimolar replacement of 20 mM NaCl with KCl) for 1 min, resulting in a 0.216 ± 0.044% evoked [^{3}H]GABA release in normoxia and 0.227 ± 0.055% in ischemia ($n = 4$ each; $p > 0.05$). Hence, the ability of the cell cultures to release [^{3}H]GABA upon stimulation by K^+ were unaffected by the ischemic conditions. When the ischemic cultures were challenged with BzATP, this agonist was one order of magnitude more potent than ATP ($EC_{50} = 120$ μM), although its maximum effect (at 1 mM) amounted to only ∼40% of the maximum ATP effect at 10 mM (Fig. 2a, ii). The induction of a stimulatory BzATP action by ischemia and the shift in the potency of ATP to the higher concentration range indicate the recruitment of $P2X_7$-like receptor activity under ischemic conditions (but see below). Apparently, ATP stimulated non-$P2X_7$ receptors at lower concentrations but became more selective to the $P2X_7$ receptor at higher concentrations (Ralevic and Burnstock 1998).

Then, we investigated the inhibitory activities of the two $P2X_7$-receptor selective antagonists oxidized ATP (300 μM) and Brilliant Blue G (1 μM), as well as that of the non-selective $P2X_7$ receptor antagonist PPADS (30 μM), on the effect of ATP. All these compounds markedly depressed the ATP (0.01–10 mM)-induced [^{3}H]GABA release both in normoxic (Fig. 2b, i) and ischemic (Fig. 2b, ii) superfusion media. It is noteworthy that Brilliant Blue G abolished a much larger fraction of the response to ATP under ischemic than under normoxic conditions, indicating that the $P2X_7$ receptor-mediated fraction is selectively increased during ischemia. Neither antagonist altered the resting release (data not shown). Finally, Brilliant Blue G (1 μM), a selective $P2X_7$ receptor antagonist, about halved the BzATP-induced [^{3}H]GABA release after ischemic stimulation (Fig. 2c) suggesting that BzATP equally activates $P2X_7$ and non-$P2X_7$ receptors at all concentrations used.

Next, we examined the possible involvement of various transmitter systems in the effect of ATP to release [^{3}H]GABA under ischemic conditions. In these experiments, the cultures were stimulated with ATP (3 mM) twice, with 10-min intervals. The net tritium release evoked by the first ATP challenge (S_1) was reproducible upon a subsequent identical stimulus (S_2), resulting in an S_2/S_1 ratio of 0.831 ± 0.053% ($n = 6$; Figs 3a and b). Tetrodotoxin (1 μM), a blocker of sodium-dependent action potentials was without effect on the S_2/S_1 ratio, confirming that ATP does not release by propagated action potentials an unknown transmitter from neighboring neurons to cause [^{3}H]GABA release. The combined blockade of ionotropic glutamate receptors of the non-NMDA- (CNQX; 10 μM) and NMDA-types (AP-5; 50 μM) were also ineffective. Similarly, the combined application of the non-selective P2Y receptor antagonist Reactive Blue 2 (10 μM) and the $P2Y_1$ receptor selective antagonist MRS 2179 (10 μM) had no effect; $P2Y_1$ receptors were described to abundantly occur on cortical neurons (Morán-Jiménez and Matute 2000). Finally, the blockade of A_1 and A_{2A} adenosine receptors by DPCPX (0.25 μM) and 8-(3-chlorostyryl)caffeine (10 μM), respectively, did not alter the response to ATP (Fig. 3b). Hence, ionotropic glutamate receptors, P2Y receptors and adenosine receptors neither mediate the stimulated [^{3}H]GABA release by ATP under ischemic conditions nor contribute to it. It appears likely that $P2X_7$ receptors situated at the GABA neurons themselves directly trigger [^{3}H]GABA release.

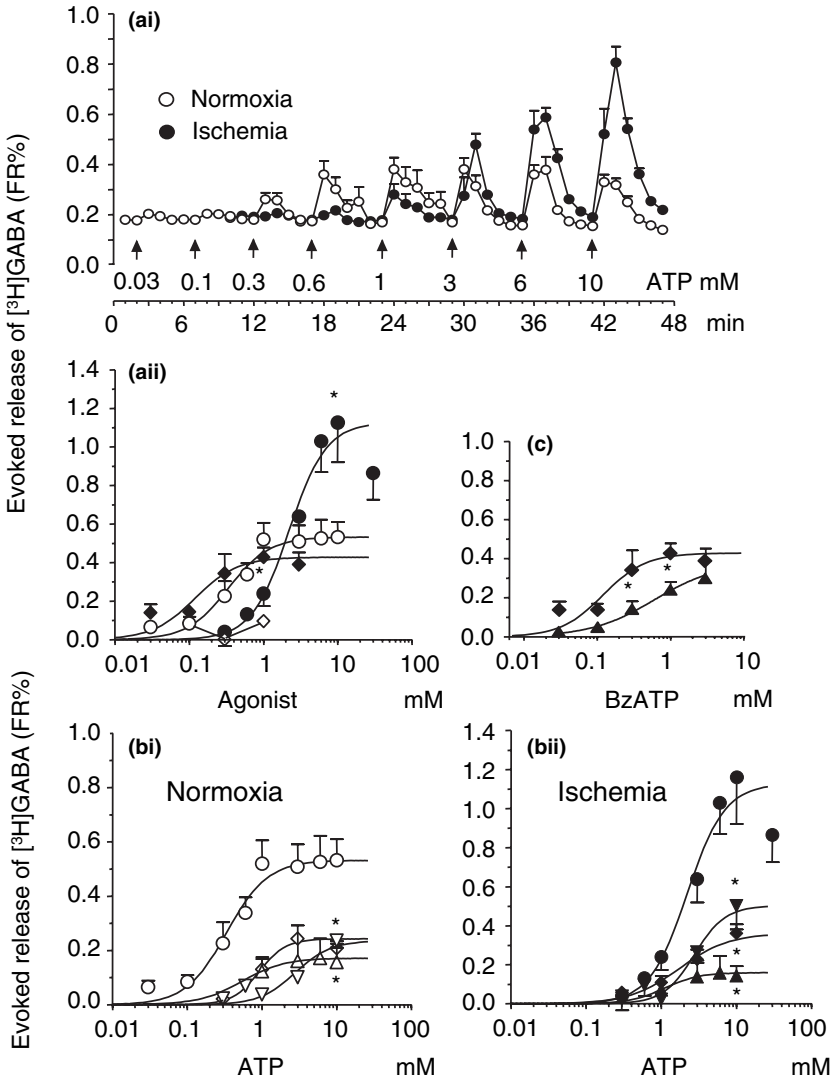

Fig. 2 Change in ATP- and 2′-3′-*O*-(4-benzoylbenzoyl)-ATP (BzATP)-induced transmitter release from cortical cell cultures of rats after ischemic stimulation; interaction with $P2X_7$ receptor antagonists. (a, i) Concentration-dependent release by ATP and BzATP of [^{3}H]GABA release; experimental protocol documenting the effect of ATP under normoxia and ischemia. After 30 min of pre-incubation, a subsequent 20-min incubation with [^{3}H]GABA, and a subsequent 15-min wash-out, 1-min samples were collected from the effluent. The cell cultures were challenged with increasing concentrations of ATP, indicated by arrows. FR% denotes the fractional release percentages. Means ± SEM of 11–13 experiments. (a, ii) Concentration–response curves of ATP and BzATP for the fractional rate of release of [^{3}H]GABA in normoxic and ischemic cell cultures. The agonist-induced [^{3}H]GABA release is calculated by the area under the curve (AUC) method from experiments similar to those shown in (a). ○, ATP in normoxia; ◇, BzATP in normoxia; ●, ATP in ischemia; ◆, BzATP in ischemia. Means ± SEM of 7–13 experiments. ATP and BzATP caused a larger maximum release in ischemic than in normoxic cultures. *$p < 0.05$; statistically significant difference from the effect of ATP or BzATP under normoxic conditions. (b) Concentration–response curves of ATP for the fractional rate of release of [^{3}H]GABA in normoxic and ischemic cell cultures. (b, i) Interaction of the $P2X_7$ receptor antagonists oxidized ATP (oxiATP), Brilliant Blue G (BBG), and pyridoxal-phosphate-6-azophenyl-2′,4′-disulfonic acid (PPADS) with ATP under normoxic conditions. ○, ATP alone; ▽, ATP + oxiATP 300 μM; △, ATP + BBG 1 μM; ◇, ATP + PPADS 30 μM. Means ± SEM of 6–12 experiments. (b, ii) Interaction of the $P2X_7$ receptor antagonists oxiATP, BBG, and PPADS with ATP under ischemic conditions. ●, ATP alone; ▼, ATP + oxiATP 300 μM; ▲, ATP + BBG 1 μM; ◆, ATP + PPADS 30 μM. The experimental protocol and the evaluation procedure was identical to that shown in Fig. 1(b, i). Means ± SEM of 4–13 experiments. *$p < 0.05$; statistically significant difference from the effect of ATP in the absence of antagonists. (c) Concentration–response curves of BzATP for the fractional release of [^{3}H]GABA in ischemic cell cultures. ◆, BzATP; ▲, BzATP + BBG. Means ± SEM of 6–8 experiments. *$p < 0.05$; statistically significant difference from the effect of BzATP alone.

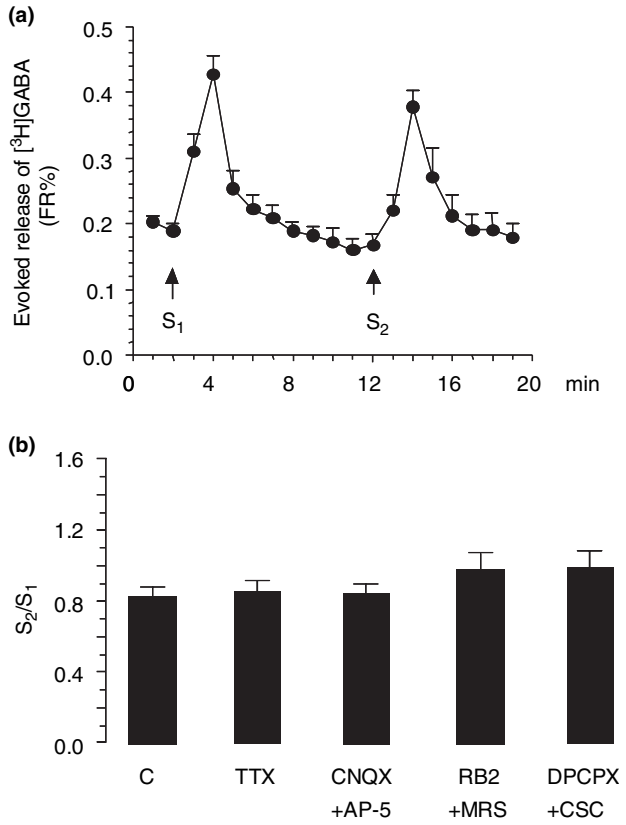

Fig. 3 Characterization of ATP-induced release of [^{3}H]GABA in ischemically treated cortical cell cultures of rats. (a) Experimental protocol for investigating the mode of action of ATP to release [^{3}H]GABA in ischemically treated cultures. Three and 13 min after starting to collect samples, 1-min ATP (3 mM) perfusions were used as indicated by the arrows (S_1, S_2), resulting in a comparable tritium outflow. (b) The presynaptic effect of ATP is independent from Na^+-dependent action potentials, and the activation of ionotropic glutamate receptors, as well as adenosine and P2Y receptors. Tetrodotoxin (TTX; 1 μM), 6-cyano-7-nitroquinoxaline-2,3-dione (CNQX; 10 μM) plus D(–)-amino-5-phosphonopentanoic acid (AP-5; 50 μM), Reactive Blue 2 (RB2; 10 μM) plus 2′-deoxy-N^6-methyladenosine 3′,5′-bisphosphate (MRS; 10 μM), and 8-cyclopentyl-1,3-dipropylxanthine (DPCPX; 0.25 μM) plus 8-(3-chlorostyryl)caffeine (CSC; 10 μM) were all applied 6 min before S_2. Means ± SEM of the S_2/S_1 ratios of 4–6 experiments.

2′-3′-*O*-(4-benzoylbenzoyl)-ATP-induced membrane currents and increase in the frequency of miniature inhibitory postsynaptic potentials of cortical neurons; interaction with Brilliant Blue G

Although in the neuronally enriched cultures used by us, astrocytes are only a minor fraction of the total cell number, and in addition β-alanin (1 mM) was included into the ACSF solution to prevent tritium uptake into glial cells (Iversen and Kelly 1975; see Materials and methods), the possibility that ATP and BzATP release GABA from astrocytes (Pannicke *et al*. 2000; Illes and Ribeiro 2004) could not be completely excluded up to this stage. Therefore, we utilized electrophysiological techniques in an attempt to discriminate between the neuronal (mostly quantal) and astrocytic (mostly non-quantal) release of GABA.

In a first series of experiments, a 140 mM KCl-containing pipette solution was used to record the membrane potential of visually identified non-pyramidal neurons, which were found to react to depolarizing current injection with action potentials (resting membrane potential, −74.8 ± 3.1 mV, $n = 5$). In all following experiments, a 140 mM CsCl-containing pipette solution was used. BzATP (300 μM) caused only negligible inward current (3.8 ± 1.6 pA), in spite of a marked response to the ionotropic glutamate receptor agonist NMDA (30 μM; 230.2 ± 37.8 pA; $n = 7$ each), which was co-applied with glycine (10 μM) and tetrodotoxin (0.3 μM). Hence, the very low sensitivity of the cortical neurons both to BzATP (300 μM) and ATP (1–10 mM; <50 pA; not shown) itself appears to exclude the presence of a considerable $P2X_7$ receptor population in untreated neurons. In addition, neither normoxic nor ischemic pre-incubation of the culture system for 30–60 min caused facilitation of the ATP- or BzATP-induced current amplitudes. As both ATP and BzATP caused only small current amplitudes if any, we did not investigate the interaction of these agonists with $P2X_7$ receptor antagonists.

In the usual 10–15-day-old cortical cultures, individual neurons became synaptically interconnected. The ACSF contained tetrodotoxin (0.5 μM), which abolishes sodium-dependent action potentials but fails to alter spontaneous quantal transmitter release. At a holding potential of −60 mV, both excitatory glutamate receptor agonists and $GABA_A$ receptor agonists induce inward currents. Therefore, CNQX (10 μM) and AP-5 (50 μM) were added to pharmacologically isolate $GABA_A$ receptor-mediated mIPSCs from contaminating NMDA and non-NMDA receptor-mediated excitatory mEPSCs.

The control frequency (1.9 ± 0.3 Hz) and amplitude (15.6 ± 1.2 pA; $n = 42$ each) of mIPSCs, respectively, could be pooled from all experiments, as there was no difference between these parameters of normoxically and ischemically treated cells. The frequency of mIPSCs slightly decreased after the first 5 min and then remained stable both after normoxic (5 min, −8.4 ± 0.9%; 10 min, −9.1 ± 1.0% change from 0 min; $n = 5$) and ischemic (5 min, 10.9 ± 2.3%; 10 min, −9.2 ± 5.5% change from 0 min; $n = 4$) incubation. It is noteworthy that the frequency increase in the normoxic cultures by BzATP (300 μM) was also stable over 10 min ($n = 5$), and Brilliant Blue G (0.3 μM) did not alter the mIPSC frequency when given alone ($n = 6$).

The $GABA_A$ receptor antagonist bicuculline (10 μM) abolished the mIPSC amplitudes measured after ischemic pre-incubation; this effect was reversible on washout (Fig. 4a, i; $n = 3$). The thereby identified spontaneous quantal release of GABA was reversibly increased by BzATP (300 μM) application for 5 min (Fig. 4a, ii). The inter-event interval distribution

of this cell was shifted towards the left, reflecting an increase of mIPSC frequency by BzATP (Fig. 4a, iii). Figure 4(a, iv) shows that the number of events at each mIPSC amplitude was augmented by BzATP. As the mean mIPSC amplitude was not altered, the data are compatible with a frequency increase (Fig. 4b, i and ii). Finally, neither the frequency nor the amplitude of mIPSCs changed within 5 min of superfusion with medium, irrespective of whether the pre-incubation was normoxic or ischemic (Fig. 4b, i and ii). The percentage potentiation of the mIPSC frequency by BzATP (300 μM), in

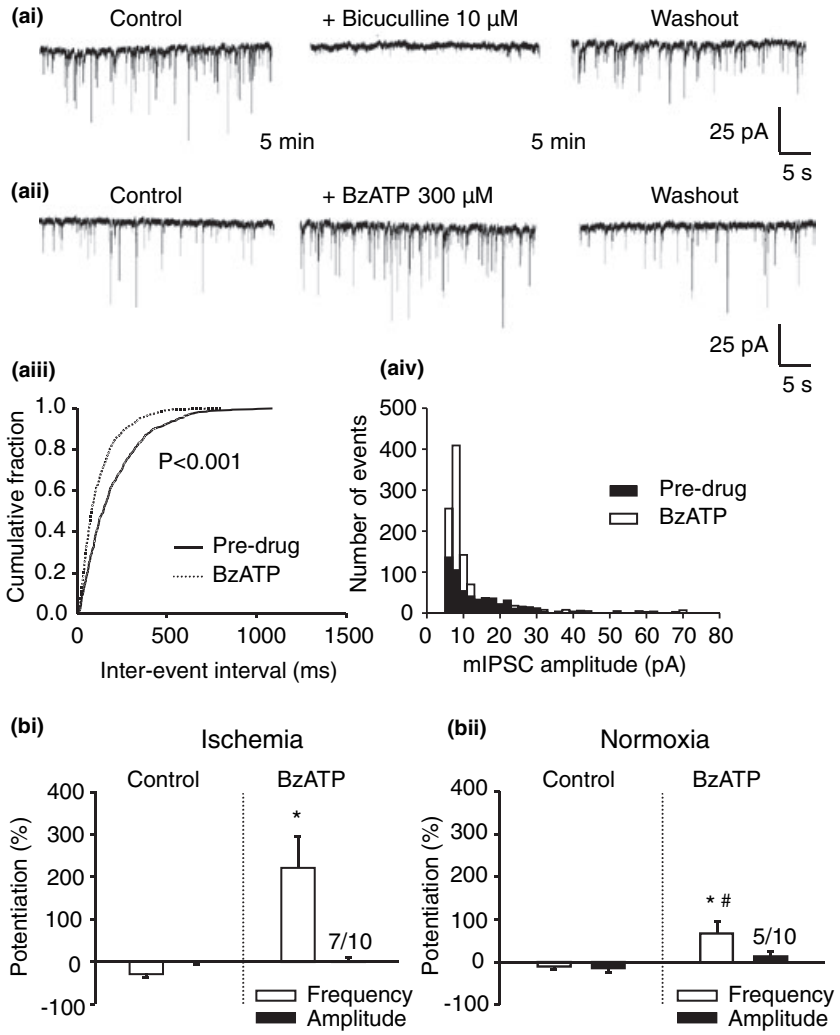

Fig. 4 Modulation by 2′-3′-*O*-(4-benzoylbenzoyl)-ATP (BzATP) of $GABA_A$ receptor-mediated mIPSCs in neurons of rat cortical cell cultures after normoxic or ischemic pre-treatment. Miniature inhibitory postsynaptic currents (mIPSCs) were recorded at a holding potential of −60 mV. 6-Cyano-7-nitroquinoxaline-2,3-dione (CNQX; 10 μM), D(−)-amino-5-phosphonopentanoic acid (AP-5; 50 μM) and tetrodotoxin (TTX; 0.5 μM) were all present in the medium. Average values of amplitude and frequency of mIPSCs were calculated during a control period of 3 min and during the last 3 min of the subsequent application of BzATP (300 μM) for 10 min in total. (a, i) Consecutive traces showing typical mIPSCs before, during and 10 min after the application of bicuculline (10 μM) in normoxic cultures. (a, ii) Consecutive traces showing typical mIPSCs before, during and 10 min after the application of BzATP (300 μM) in ischemic cultures. (a, iii) Cumulative plots of amplitude distribution before (continuous line) and after the application of BzATP (dotted line). (a, iv) The amplitude distribution before (filled bars) and after the application of BzATP (empty bars). Data obtained in the cell shown in (a, ii) were further analyzed in (a, iii) and (a, iv). (b, i) Increase of the mean frequency (empty columns) but not amplitude (filled columns) by BzATP in ischemic cells (right set of columns). The changes were expressed as percentage potentiation of the time-matching controls recorded in drug-free ACSF (left set of bars). Only seven out of 10 cells responded to the effect of BzATP. (b, ii) Increase of the mean frequency (empty columns) but not amplitude (filled columns) by BzATP in normoxic cells (right set of columns). The changes were expressed as percentage potentiation of the time-matching controls recorded in drug-free ACSF (left set of bars in b, i and b, ii; ischemia, $n = 4$; normoxia, $n = 5$). Only five out of 10 cells responded to the effect of BzATP. $p < 0.05$; statistically significant difference from zero. #$p < 0.05$; statistically significant difference from the effect of BzATP in ischemic cells.

comparison with the time-matching controls, documented a marked increase in the case of seven out of 10 ischemic cells (Fig. 4b, i) and a less pronounced increase in the case of five out of 10 normoxic cells (Fig. 4b, ii). At the same time, BzATP (300 μM) failed to alter the mean amplitude of mIPSCs when compared with the time-matching control group.

A 10-times lower concentration of BzATP (30 μM) also tended to increase the frequency of mIPSCs to a larger extent in the ischemic cells (51.1 ± 13.5%; five out of nine neurons) than in the normoxic cells (18.9 ± 2.4%; four out of 13 neurons; $p > 0.05$), although this change did not reach the level of statistical significance. In addition, there was an increase in the number of responsive cells in the ischemic cultures versus the normoxic ones (55% vs. 31%; see above). The effect of BzATP in a subset of neurons only indicates a non-homogeneity of the total GABAergic cell population investigated. The rise time from 10% to 90% of the peak current amplitude ($\tau_{10-90\%}$) and the time constant of decay (τ_{decay}) were calculated to characterize mIPSCs. The time-course of the mIPSCs, determined both in the normoxically ($\tau_{10-90\%}$, 4.2 ± 0.3 ms; τ_{decay}, 34.2 ± 1.1 ms; $n = 5$) and ischemically treated group of cells ($\tau_{10-90\%}$, 3.9 ± 0.3 ms; τ_{decay}, 35.1 ± 1.3 ms; $n = 7$), was not changed by BzATP (300 μM).

Next, we investigated whether BzATP facilitates the quantal release of GABA via $P2X_7$ receptor activation. In fact, superfusion with the $P2X_7$ receptor antagonist Brilliant Blue G (0.3 μM) for 5 min did not alter the amplitude or frequency of mIPSCs recorded from normoxically pre-treated neurons ($n = 5$; Fig. 5a, i). However, Brilliant Blue G almost abolished the facilitatory effect of BzATP (300 μM; Fig. 5a, ii and iii). As only a fraction of cortical neurons responded to BzATP application (Fig. 4), only those cells were included into this evaluation, which responded to the agonist with a frequency increase. In a further series of mIPSC measurements, superfusion with a Ca^{2+}-free ACSF (no Ca^{2+}, 1 mM EGTA) for 10 min in total, did not alter the frequency (5 min, −6.4 ± 12.9%; 10 min, 5.5 ± 8.7%; $n = 7$), but slightly decreased the amplitude (5 min, −20.0 ± 3.8%; 10 min, −26.4 ± 4.2%; $n = 7$; $p < 0.05$), when compared with the 0-min value determined in a Ca^{2+}-containing ACSF. When cortical cultures were superfused at first for 5 min with Ca^{2+}-free ACSF and subsequently for another 5 min with BzATP (300 μM) in the continuing absence of Ca^{2+}, there was no change either in the frequency (Ca^{2+}-free ACSF, −8.3 ± 8.7%; BzATP, −18.7 ± 6.7%; $n = 11$; $p > 0.05$) or in the amplitude (Ca^{2+}-free ACSF, 6.2 ± 8.6%; BzATP, 2.7 ± 11.1%; $n = 11$; $p > 0.05$) of mIPSCs, compared with the 0-min values measured in Ca^{2+}-containing ACSF.

Our experiments with mIPSCs suggest that BzATP and Brilliant Blue G interact at a presynaptic site of action. This idea was strengthened by the fact that BzATP failed to alter current responses to the $GABA_A$ receptor agonist muscimol (10 μM; Fig. 5b). The amplitude of muscimol-induced inward currents were 1094.0 ± 243.1 pA before and 1085.3 ± 293.4 pA ($n = 5$; $p > 0.05$) 6 min after superfusion with BzATP (300 μM). Hence, BzATP-sensitive $P2X_7$ receptors appear to be situated at many GABAergic nerve terminals innervating neighbouring cells in cortical cell cultures. It is unlikely that the enzymatic degradation of BzATP to Bz-adenosine, which is inactive at adenosine receptors but may hetero-exchange with intracellular adenosine (Kukley *et al*. 2004), results in the activation of facilitatory presynaptic A_{2A} receptors (for ^{3}[GABA] release see previous section). In this case, the $P2X_7$ receptor antagonistic Brilliant Blue G would certainly not be able to abolish the effect of BzATP.

As the BzATP-induced facilitation of mIPSC frequency absolutely depended on the presence of Ca^{2+} in the extracellular medium, we conclude that $P2X_7$ receptor activation triggers an exocytotic, neuronal release of GABA. The finding that BzATP failed to increase the outflow of [^{3}H]GABA (sum of the quantal and non-quantal release), although it did slightly increase the mIPSC frequency under normoxic conditions, may indicate that BzATP mostly facilitates the quantal, exocytotic secretion of transmitter (Vyskocil and Illes 1997) of GABA.

ATP- and 2′-3′-*O*-(4-benzoylbenzoyl)-ATP-induced increase in $[Ca^{2+}]_i$ of cortical neurons and astrocytes and interaction with Brilliant Blue G and pyridoxal-phosphate-6-azophenyl-2′,4′-disulfonic acid

Although $P2X_7$ receptor activation unequivocally stimulated GABA release, in neuronally enriched and ischemically primed cortical cells, it is unclear whether the release of GABA from the minor population of astrocytes is a Ca^{2+}-dependent exocytotic process contributing to the neuronal release. To answer these questions, experiments were designed to measure the cytosolic-free Ca^{2+} concentration by the fura-2 technique; the fluorescence ratio (340/380 nm) was taken as a relative measure of free $[Ca^{2+}]_i$ (for absolute values see the Materials and methods section).

The responses to high external K^+ (50 mM; cell depolarization and subsequent opening of voltage-gated Ca^{2+} channels), as well as to ATP (300 μM) and BzATP (300 μM) were tested on neuronal somata and proximal dendrites, as well as on astrocytes. We have chosen astrocyte-free areas for the determination of $[Ca^{2+}]_i$ in dendrites. Measurements were made either in untreated cultures, or in normoxically/ischemically pre-incubated cultures (Fig. 6). Whereas in untreated cultures, high external K^+ caused a pronounced $[Ca^{2+}]_i$ signal in all neuronal cell bodies tested, the responses in dendrites and astrocytes became gradually smaller (Fig. 6a; Bennett *et al*. 2003). By contrast, the ATP- and BzATP-induced $[Ca^{2+}]_i$ responses were very small in neuronal somata, but considerably larger in dendrites and especially in astrocytes.

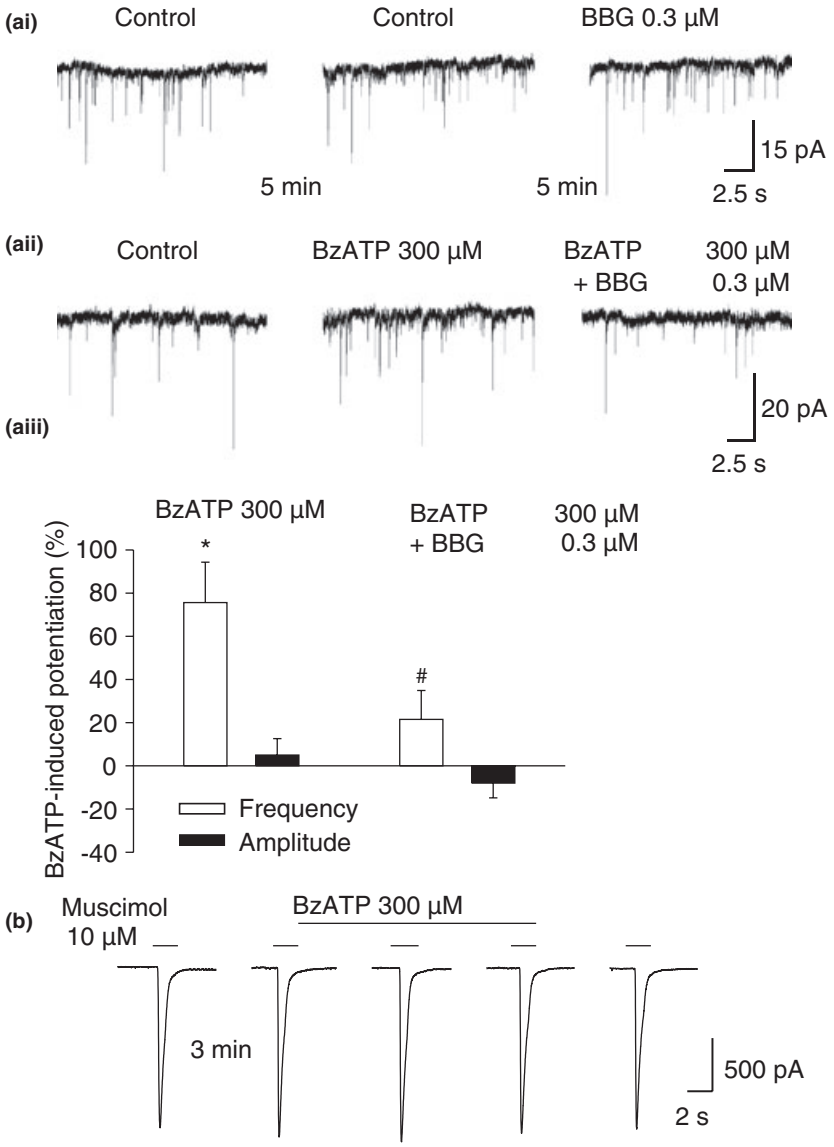

Fig. 5 Modulation by 2′-3′-*O*-(4-benzoylbenzoyl)-ATP (BzATP) of $GABA_A$ receptor-mediated miniature inhibitory postsynaptic currents (mIPSCs) in neurons of rat cortical cell cultures after normoxic pre-treatment and interaction with Brilliant Blue G (BBG); no effect of BzATP on the current response to muscimol. mIPSCs were recorded at a holding potential of −60 mV. 6-Cyano-7-nitroquinoxaline-2,3-dione (CNQX; 10 μM), D(−)-amino-5-phosphonopentanoic acid (AP-5; 50 μM) and tetrodotoxin (TTX; 0.5 μM) were all present in the medium. Average values of amplitude and frequency of mIPSCs were calculated during a control period of 3 min, during the last 3 min of the subsequent application BBG (0.3 μM) for 5 min, as well as during the last 3 min of further superfusion with BBG alone or in combination with BzATP (300 μM) for a further 5 min. (a, i *and* ii) Consecutive traces showing typical experiments in two individual cells. (a, iii) Increase of the mean frequency (empty columns) but not amplitude (filled columns) of mIPSCs by BzATP in normoxic cells; no increase of mIPSC frequency in the presence of BBG ($n = 7$; see a, ii). The changes were expressed as percentage potentiation of the time-matching controls recorded in drug-free ACSF for 10 min and in ACSF containing BBG (0.3 μM) for an additional 5 min ($n = 5$; see a, i). *$p < 0.05$; statistically significant difference from zero. #$p < 0.05$; statistically significant difference from the effect of BzATP in the absence of BBG. (b) Effect of BzATP (300 μM) on the inward current induced by muscimol (10 μM) locally superfused for 2 s with 3-min intervals. BzATP was superfused for 6 min during two consecutive muscimol applications. Typical recording out of five similar ones.

In normoxic and ischemic cell cultures, the responses of the neuronal cell bodies to high external K^+ tended to decrease when compared with those of untreated preparations, although this change did not reach the level of statistical significance (Figs 6a–c). Moreover, the ATP-induced $[Ca^{2+}]_i$ transients were clearly depressed by the normoxic/ischemic pre-incubation in astrocytes. The measurements on dendrites indicated no changes after

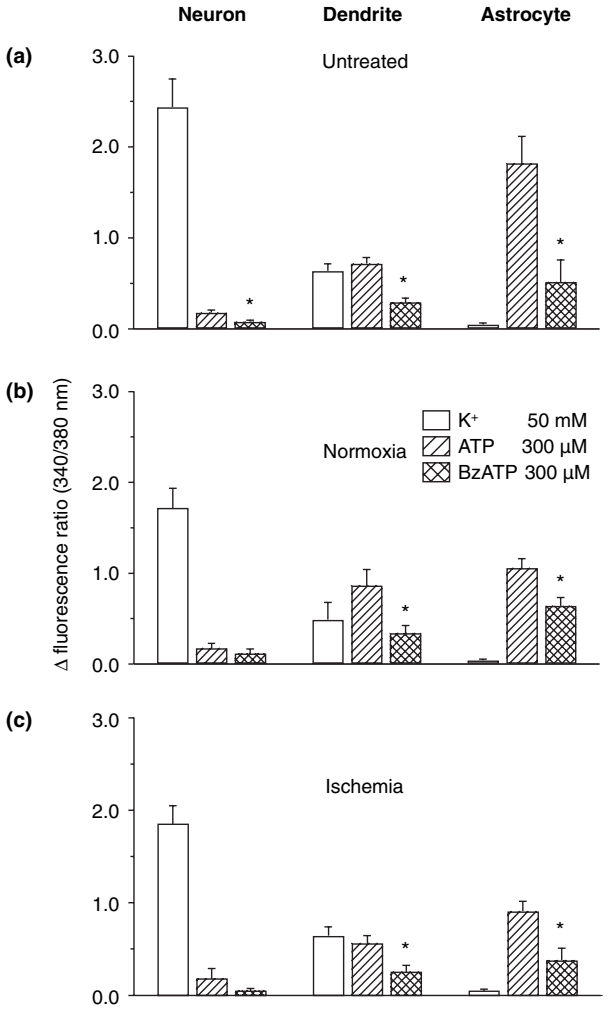

Fig. 6 Increase of $[Ca^{2+}]_i$ caused by a high potassium-containing external medium, ATP or 2′-3′-*O*-(4-benzoylbenzoyl)-ATP (BzATP) in cell somata and proximal dendrites of neurons as well as in astrocytes of cortical cell cultures of rats. Cells were loaded with the Ca^{2+} indicator fura-2/AM (5 μM). The fluorescence ratio (340/380 nm) was taken as a measure of $[Ca^{2+}]_i$. Cells were depolarized by a 50 mM K^+-containing extracellullar medium (empty columns) for 3 s every 12 min. ATP (300 μM; hatched columns) or BzATP (300 μM; cross-hatched columns) were applied for 10 s every 12 min. Responses of non-treated controls (a, $n = 12$) as well as normoxically (b, $n = 9$) and ischemically (c, $n = 11$) pre-incubated cultures are shown. *$p < 0.05$; significant difference from ATP. There is no significant difference within the K^+-, ATP- and BzATP-induced $[Ca^{2+}]_i$ responses between (a) (b) and (c), with the exception of the ATP signals in the astrocytes of normoxically and ischemically incubated cultures, which are smaller than the respective signals under normal conditions ($p < 0.05$).

normoxic/ischemic pre-incubation. Finally, ischemia in comparision with normoxia did not alter the responses to either ATP or BzATP in either cell-type. Hence, all these results unequivocally argue against an up-regulation of P2 receptor function in the somata and dendrites of neurons as well as in astrocytes.

In the following experiments, we tried to find out which type of P2 receptor is situated at dendrites and astrocytes of cortical cultures. By definition, P2X receptors are ligand-gated cationic channels and therefore allow the passage of Ca^{2+} from the external medium, whereas P2Y receptors may be coupled to G proteins, which release Ca^{2+} from its intracellular stores via inositol 1,4,5-trisphosphate (Abbracchio and Burnstock 1994; Illes *et al*. 2000). Therefore, we roughly characterized the P2 receptors by measuring the increase of $[Ca^{2+}]_i$ in proximal dendrites of cortical neurons and astrocytes in response to ATP (300 μM), both in the presence and absence of external Ca^{2+} or cyclopiazonic acid (Fig. 7a). The $[Ca^{2+}]_i$ transients of the neuronal cell bodies were not evaluated, because of their very small amplitudes (Figs 6a–c). Cyclopiazonic acid inhibits the Ca^{2+}-ATPase of the endoplasmatic reticulum and thereby depletes this intracellular Ca^{2+} pool. A Ca^{2+}-free medium (plus 1 mM EGTA) and cyclopiazonic acid (10 μM) both depressed the ATP-evoked increase of $[Ca^{2+}]_i$, indicating that proximal dendrites and astrocytes possess a mixed P2X/P2Y receptor population (Fig. 7a).

As our primary goal was to prove or disprove an ischemia-induced supersensitivity of $P2X_7$ receptors in the investigated cells, we also utilized the $P2X_7$ receptor preferential agonist BzATP (300 μM) in interaction with the selective and non-selective $P2X_7$ receptor antagonists Brilliant Blue G (1 μM) and PPADS (30 μM), respectively (Ralevic and Burnstock 1998; Jiang *et al*. 2000; Fig. 7b). Brilliant Blue G failed to depress the increase of $[Ca^{2+}]_i$ caused by either ATP or BzATP and both following normoxic and ischemic stimulation (Fig. 7b, i). By contrast, PPADS markedly inhibited $[Ca^{2+}]_i$ responses to both agonists in dendrites as well as astrocytes (Fig. 7b, ii and iii). Although under normoxic conditions PPADS depressed the response to ATP in astrocytes with a slightly lower efficiency than in the proximal dendrites, PPADS was absolutely equipotent in blocking agonist effects under normoxia and ischemia in neurons and astrocytes.

In conclusion, the measurement of $[Ca^{2+}]_i$ transients in proximal dendrites and astrocytic cell bodies failed to support the notion that $P2X_7$ receptor activation initiates a Ca^{2+}-dependent release of GABA from these cellular sites. The quantal release of transmitters (e.g. glutamate) from astrocytes has been shown to be triggered by an increase of the intracellular Ca^{2+} concentration leading to the activation of the exocytotic machinery (Pasti *et al*. 2001; Nedergaard *et al*. 2002). However, this was certainly not the case under the present experimental conditions.

Discussion

A marked decline of intracellular ATP levels with a concomitant efflux of ATP into the extracellular space has been shown to occur in the rat brain during the first minutes after oxygen depletion *in vivo* (Hisanaga *et al*. 1986; Volonte *et al*. 2003). Cerebral ischemia also activated microglial cells

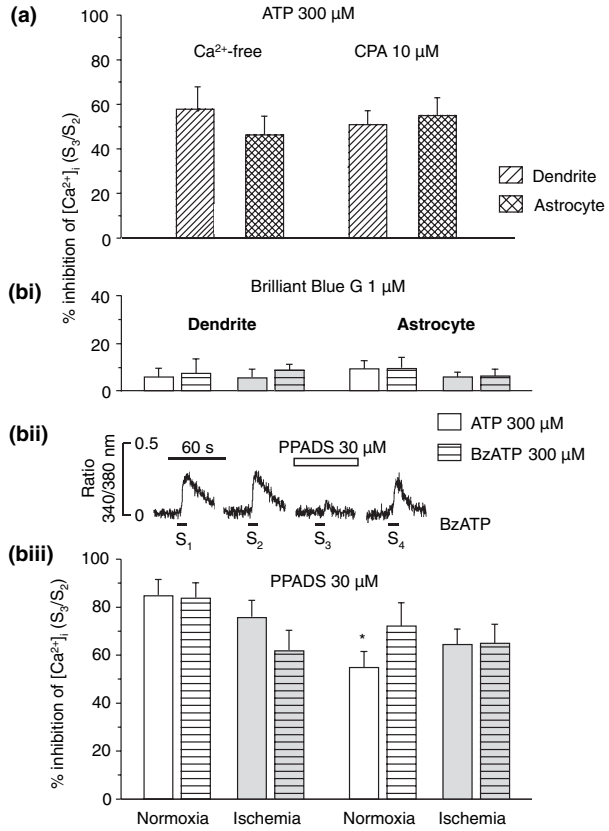

Fig. 7 Characterization of P2 receptor types on proximal dendrites and astrocytes by measurements of $[Ca^{2+}]_i$ in cortical cell cultures of rats. Cells were loaded with the Ca^{2+} indicator fura-2/AM (5 μM). The fluorescence ratio (340/380 nm) was taken as a measure of $[Ca^{2+}]_i$. (a) Effects of a Ca^{2+}-free medium (containing 1 mM EGTA) or cyclopiazonic acid (CPA; 10 μM) on ATP-induced Ca^{2+} responses in dendrites (hatched columns) and astrocytes (cross-hatched columns). The cultures were continuously superfused with physiological solution and stimulated four times (S_1–S_4) with ATP (300 μM) for 10 s every 12 min (b, ii). A Ca^{2+}-free medium or cyclopiazonic acid were superfused for 10 min both before and during the stimulation with ATP (S_3). Drug effects were evaluated as the percentage change of the ATP-induced signal at S_3 versus the second control signal at S_2. (b) Effect of the $P2X_7$ receptor antagonists Brilliant Blue G (1 μM; b, i) and PPADS (30 μM; b, ii *and* iii) on the $[Ca^{2+}]_i$ responses caused by ATP (300 μM; empty columns) and 2′-3′-*O*-(4-benzoylbenzoyl)-ATP (BzATP; 300 μM; dashed columns) in neuronal dendrites and astrocytes. The pre-incubation condition (normoxia or ischemia) is indicated by the absence or presence of grey shading. Stimulation by ATP and BzATP, superfusion with the antagonists, and the evaluation of drug effects was identical to that described in (a). $^*p < 0.05$; significant difference from the effect of ATP (300 μM) in dendrites after normoxic incubation. Means ± SEM of $n = 4$–7 experiments in each column. Typical recording made on a proximal dendrite from an ischemically treated cell culture (b, ii).

as well as increased the expression of $P2X_7$ receptors on this cell type (Collo *et al*. 1997; Morigiwa *et al*. 2000). In addition, both mechanical (Franke *et al*. 2001) and ischemic damage (Franke *et al*. 2004) of the rat brain likewise led to the expression of previously absent $P2X_7$ receptors on astrocytes and neurons.

Useful model systems to study ischemic alterations under *in vitro* conditions are cortical cell cultures in which hypoxia was caused by substituting oxygen in the medium by argon (Günther *et al*. 2002; Reinhardt *et al*. 2003). Argon has been reported to rapidly displace residual oxygen from the incubation medium and thereby to lead to a much faster decline of cellular oxygen levels than exposure to nitrogen (Kusumoto *et al*. 1996). The duration of the ischemic stimulus was 30–60 min, because already a 30-min period was shown previously to markedly decrease the ATP/ADP ratio regarded as a measure of the metabolic limitation (Günther *et al*. 2002; Reinhardt *et al*. 2003).

In previous *in vivo* experiments, 4 days after MCAO a marked up-regulation of $P2X_7$ receptor expression was observed both by immunohistochemistry and western-blot analysis (Franke *et al*. 2004). On a first glance, these results appear to contrast with our data demonstrating no change of $P2X_7$ receptor IR by ischemia at either neurons or astrocytes. We assume that the ischemic stimulus in the long term leads to the synthesis of new $P2X_7$ receptors, although in the short term an increased functional response may prevail. Under *in vivo* conditions, cortical neurons and astrocytes of the intact brain were found to be devoid of $P2X_7$ receptor IR (Franke *et al*. 2004). Such an immunoreactivity was, however, present on cultured astrocytes kept in a serum-containing medium and was up-regulated on subsequent serum deprivation. It is suggested that the culturing procedures may initiate the synthesis of this ‘suicide receptor’ as an early apoptotic signal (North 2002).

Recently, two publications described the presence of $P2X_7$ receptor IR in the hippocampus of $P2X_7^{-/-}$ mice, and therefore seriously questioned the validity of the assumedly selective antibodies (Kukley *et al*. 2004; Sim *et al*. 2004). However, $P2X_7$ receptor IR was missing in the retinal ganglion cell layer of $P2X_7^{-/-}$ mice, when compared with their wild-type controls (Franke *et al*. 2005), suggesting a region-specific expression of immunoreactivity for neuronal $P2X_7$ receptors in the brain of the knockouts. In addition, in granule cells cultured from cerebella of $P2X_7^{-/-}$ mice, functional $P2X_7$-like receptors were found that keep some of the properties of the genuine receptor (Sanchez-Nogueiro *et al*. 2005). These receptors may represent splice variants containing the epitope (e.g. Arg-576 to Tyr-595 in their C-terminus) against which antibodies were generated, but being devoid of amino acid sequences responsible for important receptor functions (Cheewatrakoolpong *et al*. 2005). Hence, the general denial to utilize antibodies for identifying $P2X_7$ receptor protein in the brain and the refusal to accept the existence of neuronal $P2X_7$ receptors appears to be at the present time premature.

The main finding of the present study is that neuronal presynaptic $P2X_7$ receptors exhibited an increased response to ATP and BzATP after ischemic stimulation in spite of no changes in $P2X_7$ mRNA and IR. As our culturing conditions favored an enrichment of GABAergic interneuron-like cells (Fischer *et al*. 2002), we measured the release of previously incorporated [^{3}H]GABA in response to increasing concentrations of ATP and BzATP mostly from this cell population. Although BzATP had no effect in normoxic cell cultures, it became active in ischemically stimulated cell cultures. The maximum effect of ATP to release [^{3}H]GABA was markedly potentiated by ischemia, although at the same time a rightward shift of the curve occurred. Because ATP is a non-selective agonist at all P2X (and many P2Y) receptor types, and only high concentrations of the agonist activate $P2X_7$ receptors, it appears that ischemia potentiates the $P2X_7$ receptor function, but depresses the effect of some other release-enhancing P2 receptors. The non-selective antagonist PPADS and the selective $P2X_7$ antagonists Brilliant Blue G and oxidized ATP, all inhibited the effect of ATP. The results with Brilliant Blue G are especially convincing, as a much larger fraction of the release was abolished by the antagonist after the ischemic, in comparison with the normoxic pre-incubation. It is noteworthy that Brilliant Blue G at 1 μM has been described not to considerably interfere with any of the recombinant P2X receptors expressed in HEK293 cells (Jiang *et al*. 2000). In addition, Brilliant Blue G at this concentration did not alter the $[Ca^{2+}]_i$ transients caused by ATP/BzATP either in dendrites or astrocytes (see below), excluding the blockade of P2X receptors of the non-$P2X_7$ subtypes by this antagonist.

Hence, the question arises, whether $P2X_7$ receptors are situated at the GABAergic neuronal terminals themselves or at neighboring neurons/astrocytes. The measurement of intrasynaptosomal $[Ca^{2+}]_i$ transients in pinched off synaptic terminals confirmed an influx of Ca^{2+} via $P2X_7$ receptor channels (Lundy *et al*. 2002; Miras-Portugal *et al*. 2003). Alternative possibilities are that the release of GABA occurs from astrocytes (Pannicke *et al*. 2000), or that neighboring excitatory neurons bearing $P2X_7$ receptors indirectly stimulate the GABAergic neuronal activity. However, an indirect mode of action by a transmitter or neuromodulator is most unlikely, as this compound does not act via ionotropic glutamate receptors (CNQX, AP-5), P2Y receptors (Reactive Blue 2, MRS 2179) or A_1 and A_{2A} receptors (DPCPX, CSC), as proved by the respective antagonists. An action potential-dependent release of a transmitter from neighbouring neurons could be discarded, as tetrodotoxin failed to depress the increase in the ATP-induced [^{3}H]GABA release after ischemia. Based on these data as a whole, a direct stimulation of GABAergic nerve terminals by their $P2X_7$ receptor population is the most likely mechanism of action.

The membrane currents caused by high concentrations of the non-selective agonist ATP or of the $P2X_7$ receptor preferential agonist BzATP were similar when measured after normoxic and ischemic pre-incubation. By contrast, BzATP increased the frequency of the GABAergic mIPSCs in a manner absolutely depending on external Ca^{2+}; this suggests that in the network formed by cultured interneurons, the spontaneous quantal release of the transmitter GABA is enhanced by presynaptic $P2X_7$ receptors situated at the axon terminals themselves. A presynaptic site of action of BzATP is favored by the change in mIPSC frequency without a corresponding change in amplitude and by the failure of BzATP to interfere with the postsynaptic effect of the $GABA_A$ receptor agonist muscimol.

ATP and BzATP induced similar increases in neuronal (somatic/dendritic) and astrocytic $[Ca^{2+}]_i$ transients after pre-incubation with normoxic medium or with hypoxic and glucose-free medium. The strong depression of the $[Ca^{2+}]_i$ response to the two agonists by the non-selective P2X/P2Y receptor antagonist PPADS in the neuronal dendrites as well as in the astrocytes confirmed the participation of P2 receptors. However, the selective $P2X_7$ receptor antagonist Brilliant Blue G did not alter the ATP and BzATP effects excluding the involvement of this receptor subtype. Therefore, these data do not support a Ca^{2+}-dependent exocytotic GABA release initiated by $P2X_7$ receptors situated at the proximal dendrites of neurons or at the cell bodies of astrocytes (see Results; Pasti *et al*. 2001; Nedergaard *et al*. 2002).

In conclusion, neuronal $P2X_7$ receptors may release glutamate (hippocampus, Deuchars *et al*. 2001; Sperlágh *et al*. 2002) and, in consequence (Sperlágh *et al*. 2002) or independently of this effect (present study), GABA. It is necessary to emphasize that an unequivocal evidence for the involvement of the $P2X_7$ receptor subtype in these processes is still lacking. Complicating factors are the unavailability of absolutely selective agonists, antagonists, and possibly also antibodies for $P2X_7$ receptors. However, based on the multitude of experimental approaches used by us, we are quite confident that ATP or BzATP by acting on neuronal $P2X_7$ receptors in fact release GABA and that this effect is greatly enhanced after a preceding ischemic stimulus. It is suggested that an augmentation of the excitatory glutamatergic system may facilitate the ischemia-induced neuronal damage, whereas the augmentation of the inhibitory GABAergic system may be neuroprotective; the sum of these two opposing influences may determine the infarct size. The existence of a massive GABAergic innervation in the parietal cortex may explain the finding that in $P2X_7$ receptor-deficient mice or after the application of $P2X_7$ receptor antagonists, MCAO caused an infarct size of similar magnitude than in untreated wild-type mice (Le Feuvre *et al*. 2003). However, after mechanical damage to the spinal cord, $P2X_7$ receptor antagonists significantly improved both functional recovery and diminished cell death in the peritraumatic zone (Wang *et al*. 2004). This may be due to the scarce GABAergic innervation of the spinal cord.

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