

PURINERGIC MODULATION OF GLUTAMATE RELEASE UNDER ISCHEMIC-LIKE CONDITIONS IN THE HIPPOCAMPUS

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Abstract—The aim of the present study was to explore whether endogenous activation of different purine receptors by ATP and adenosine contributes to or inhibits excess glutamate release evoked by ischemic-like conditions in rat hippocampal slices. Combined oxygen–glucose deprivation (OGD) elicited a substantial, $[Ca^{2+}]_o$ -independent release of $[^3H]$ glutamate, which was tetrodotoxin (1 μM)-sensitive and temperature-dependent. The P2 receptor antagonist pyridoxal-phosphate-6-azophenyl-2',4'-disulfonic acid (PPADS, 0.1–10 μM), and the selective P2X₇ receptor antagonist Brilliant Blue G (1–100 nM), decreased OGD-evoked $[^3H]$ glutamate efflux indicating that endogenous ATP facilitates ischemia-evoked glutamate release. The selective A₁-receptor antagonist 1,3-dipropyl-8-cyclopentylxanthine (DPCPX, 0.1–250 nM) and the selective A_{2A} receptor antagonists 4-(2-[7-amino-2-)-2-furyl(triazolo-[1,3,5]triazin-5-ylamino)ethyl)phenol (ZM241385, 0.1–20 nM) and 7-(2-phenylethyl)-5-amino-2-(2-furyl)-pyrazolo-[4,3-e]-1,2,4-triazolo[1,5-c]pyrimidine (SCH58261, 2–100 nM) decreased OGD-evoked $[^3H]$ glutamate efflux, indicating that endogenous adenosine also facilitates glutamate release under these conditions. The effect of DPCPX and ZM241385 was reversed, whereas the action of P2 receptor antagonists was potentiated by the selective ecto-ATPase inhibitor 6-N,N-diethyl-D- β , γ -dibromomethyleneATP (ARL67156, 50 μM). The binding characteristic of the A_{2A} ligand $[^3H]$ CGS21680 to hippocampal membranes did not change significantly in response to OGD. Taken together these data suggest that while A₁ receptors might become desensitized, A_{2A} and P2X receptor-mediated facilitation of glutamate release by endogenous ATP and its breakdown product adenosine remains operational under long-term OGD. Therefore the inhibition of P2X/

A_{2A} receptors rather than the stimulation of A₁ adenosine receptors could be an effective approach to attenuate glutamatergic excitotoxicity and thereby counteract ischemia-induced neurodegeneration. © 2007 IBRO. Published by Elsevier Ltd. All rights reserved.

Key words: P2 receptor, A_{2A} receptor, ischemia, release, receptor binding, hippocampus.

Increased extracellular glutamate levels and subsequent excitotoxicity are thought to be one of the major pathological factors leading to neuronal death upon a variety of neurodegenerative diseases having different origins. These may include stroke, trauma, and epilepsy (Leker and Shohami, 2002; Smith, 2004). Therefore in theory the inhibition of glutamate release is a reliable strategy to intervene in neurodegeneration. However, previous clinical trials with antagonists and modulators of glutamate receptors were largely disappointing so far, despite their powerful neuroprotective activity found in various *in vitro* and *in vivo* animal models. This is partly due to their unwanted central side effects caused by the global blockade of glutamatergic transmission in the brain and partly the highly complex nature of ischemic pathology, which involves numerous parallel and interdependent mechanisms on sub-cellular, cellular and system levels (Lee et al., 1999). Therefore intervention on individual target sites is insufficient to influence powerfully the final outcome. Nevertheless, therapeutic approaches targeting selectively pathological, excitotoxic glutamate release, but leaving unaffected glutamate release evoked by normal neuronal activity might be still a promising alternative, especially if they also target additional mechanisms critical for neuronal survival (Leker and Shohami, 2002; Smith, 2004).

Both ATP and adenosine are important neuromodulators in the hippocampus. Endogenous ATP is released frequency-dependently from *in vitro* hippocampal slices upon low and high frequency electrical stimulation (Wieraszko et al., 1989; Cunha et al., 1996a). Glutamate releasing nerve terminals of the hippocampus express functional A₁ (Corradetti et al., 1984) and A_{2A} (Caciagli et al., 1995; Cunha et al., 1997; Lopes et al., 2002) adenosine receptors as well as P2Y₁, P2Y₂, P2Y₄ (Rodrigues et al., 2005), P2X₁, P2X_{2/3}, P2X₃, (Rodrigues et al., 2005), P2X₂ (Khakh et al., 2003) and P2X₇ (Sperlágh et al., 2002; Fellin et al., 2006) nucleotide receptors. Under normoxic conditions, the activation of A₁ (Corradetti et al., 1984) and P2Y receptors (Rodrigues et al., 2005) inhibits, whereas the activation of A_{2A} and all types of P2X receptors facilitates glutamate release (Caciagli et al., 1995; Lopes et al., 2002;

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Abbreviations: ADA, adenosine deaminase; ANOVA, analysis of variance; ARL67156, 6-N,N-diethyl-D- β , γ -dibromomethyleneATP; AUC, area-under-the-curve; BBG, Brilliant Blue G; CGS15943, 9-chloro-2-(2-furyl)-(1,2,4)triazolo(1,5-c)quinazolin-5-amine; CGS21680, 2-(4-(2-p-carboxyethyl)phenylamino)-5-N-ethylcarbox-amidoadenosine; CSC, 8-(3-chlorostyryl)-caffeine; DPCPX, 1,3-dipropyl-8-cyclopentylxanthine; EC, adenylate energy charge; HPLC, high pressure liquid chromatography; HPLC-UV, high pressure liquid chromatography combined with ultraviolet detection; MCAo, middle cerebral artery occlusion; OGD, combined oxygen–glucose deprivation; PPADS, pyridoxal-phosphate-6-azophenyl-2',4'-disulfonic acid; SCH58261, 7-(2-phenylethyl)-5-amino-2-(2-furyl)-pyrazolo-[4,3-e]-1,2,4-triazolo[1,5-c]pyrimidine; TTX, tetrodotoxin; VSSC, voltage sensitive sodium channels; ZM241385, 4-(2-[7-amino-2-)-2-furyl(triazolo[2,3-a]-[1,3,5]triazin-5-ylamino)ethyl)phenol.

Sperlagh et al., 2002; Khakh et al., 2003; Marcoli et al., 2003; Rodrigues et al., 2005), as described by neurochemical and electrophysiological experiments. The facilitation of glutamate release by endogenous activation of P2X receptors generates a tonic current in CA1 pyramidal neurons (Fellin et al., 2006) and contributes to carbachol-induced network oscillations (Khakh et al., 2003). Tonic activation of A₁ and A_{2A} receptors also has profound effects on neuronal excitability, and has been implicated in plasticity events such as long-term potentiation and heterosynaptic depression (de Mendonca and Ribeiro, 1997; Dunwiddie and Masino, 2001; Serrano et al., 2006). Adding further complexity to purinergic signaling in the hippocampus, the stimulation of P2 receptors releases adenosine which then leads to the activation of A_{2A} receptors and facilitation of long-term potentiation (Almeida et al., 2003). Nevertheless, the exact conditions, under which the activation of purinergic receptors by endogenous ligands gains significance remain to be identified.

One possibility is that purinergic receptors remain relatively silent under physiological conditions, and change their pattern of activity when pathological signals and cellular damage provide a purine-rich extracellular milieu for their increased activation. Indeed, purines are released in large amounts to the extracellular space during a wide variety of pathological stimuli, including metabolic distress and cellular injury. Whereas the massive release of adenosine under hypoxia, hypercapnia, glucose and energy deprivation is well documented (e.g. Latini et al., 1999b; Dulla et al., 2005, for further references see Latini and Pedata, 2001; Pearson et al., 2003) it is now also recognized that extracellular ATP also accumulates under similar conditions *in vitro* and *in vivo* (Lutz and Kabler, 1997; Juranyi et al., 1999; Melani et al., 2005; Frenguelli et al., 2007).

Another body of data indicates that the expression and function of purine receptors change activity-dependently upon pathological conditions. P2X7 receptor immunoreactivity is up-regulated following ischemia in the middle cerebral artery occlusion model (MCAo) earlier in neurons (Franke et al., 2004) and in a later phase in microglial cells as well (Collo et al., 1997). P2X2 and P2X4 receptors are also up-regulated following oxygen–glucose deprivation in organotypic hippocampal and corticostriatal slice cultures (Cavaliere et al., 2003), whereas A₁ adenosine receptors appear to lose their sensitivity upon prolonged energy deprivation (Coelho et al., 2006). Previously we showed that activation of P2X7 receptors elicits glutamate release in the rat (Sperlagh et al., 2002) and mouse (Papp et al., 2004b) hippocampus. Furthermore, we revealed that the functional responsiveness of P2X7 receptors expressed on cerebrocortical neurons is increased upon combined oxygen and glucose deprivation both in electrophysiological recordings and in transmitter release experiments (Wirkner et al., 2005). However, it remains unclear, whether P2X7 and other P2 receptors are indeed activated by endogenous purines under similar conditions. Therefore we used the rat hippocampal slices preloaded with [³H]glutamate to study the participation of various purine

receptors in the shaping of extracellular glutamate levels under combined oxygen–glucose deprivation.

EXPERIMENTAL PROCEDURES

All studies were conducted in accordance with the principles and procedures outlined in the *NIH Guide for the Care and Use of Laboratory Animals* revised in 1996 and were approved by the local Animal Care Committee of the Institute of Experimental Medicine (Hungary). All efforts were made to minimize both the suffering and the number of animals used.

Neurotransmitter release experiments from rat hippocampal slices

[³H]Glutamate release experiments were carried out as previously described (Sperlagh et al., 2002). Male Wistar rats (140–160 g, bred at the local animal house) were decapitated and the brain was quickly put into ice-cold Krebs solution (in mM: NaCl 115, KCl 3, KH₂PO₄ 1.2, MgSO₄ 1.2, CaCl₂ 2.5, NaHCO₃ 25, glucose 10, pH 7.4), oxygenated with 95% O₂+5% CO₂. Both hippocampi were rapidly dissected and 400- μ m-thick slices were cut transversely with a Mcllwain tissue chopper (Bachofner, Reitingen, Germany) and incubated in 1 ml oxygenated Krebs solution containing 10 μ Ci L-[G-³H]glutamate (0.2 μ M, specific activity 49 Ci/mmol) for 45 min at 37 °C. After incubation, the slices were rinsed three times with 6 ml Krebs solution, and each slice was transferred to one of four polypropylene tissue chambers, and was perfused continuously with 95% O₂+5% CO₂-saturated Krebs solution (flow rate: 0.6 ml/min). To minimize the spontaneous firing of CA1 and CA3 pyramidal cells the bath temperature during superfusion was kept at 32 °C. Upon termination of the 60-min preperfusion period, 3-min samples of the effluent were collected and assayed for [³H]glutamate. Ischemic-like conditions were simulated by combined oxygen–glucose deprivation (OGD): the perfusion fluid was replaced by Krebs solution lacking glucose and carefully saturated with 95% N₂+5% CO₂. Since a closed superfusion system was used, there was no leakage of either oxygen or other gas from the perfusion solution. In some experiments antagonists (pyridoxalphosphate-6-azophenyl-2',4'-disulfonic acid (PPADS), Brilliant Blue G (BBG), 1,3-dipropyl-8-cyclopentylxanthine (DPCPX), 4-(2-[7-amino-2-)-furyl(triazolo{2,3-a}-[1,3,5]triazin-5-ylamino)ethyl)phenol (ZM241385), 7-(2-phenylethyl)-5-amino-2-(2-furyl)-pyrazolo-[4,3-e]-1,2,4-triazolo[1,5-c]pyrimidine (SCH58261) or tetrodotoxin (TTX)) were preperfused in the absence or presence of the ecto-ATPase inhibitor 6-N,N-diethyl-D- β , γ -dibromomethyleneATP (ARL67156), or the bath was fast-cooled to 12 °C as described earlier (Vizi and Sperlagh, 1999) by the Frigomix R thermoelectric device (Braun Instruments, Darmstadt, Germany) 30 min before the beginning of the sample collection period, and all procedures lasted till the end of the sample collection period. In other experiments the perfusion solution was replaced with Ca²⁺-free Krebs solution supplemented with 1 mM EGTA from the beginning of the preperfusion period and thereafter.

Measurement of sample radioactivity

The radioactivity released from the preparations was measured with a Packard Tricarb 1900 liquid scintillation spectrometer (Canberra, Australia), which is equipped with Dynamic Color Corrected DPM Option providing absolute activity (DPM) calculation and correction for all kinds of quenching. A 0.5 ml aliquot of the perfusate sample was added to 2 ml of liquid scintillation fluid (Packard Ultima Gold) and counts were measured. For determining the radioactivity remaining in the tissue hippocampal slices were weighed and homogenized, and the radioactivity was extracted with 10% trichloroacetic acid. In our previous studies using similar protocols (Nakai et al., 1999; Kofalvi et al., 2003, 2005), it

was shown by high pressure liquid chromatography (HPLC) analysis that [^3H]glutamate is a good indicator of endogenous release of glutamate, therefore tritium release was used as a marker of glutamate release, but for the sake of simplicity we refer to this release as [^3H]glutamate release. The release of tritium was calculated in Bq/g and expressed as the percentage of the amount of radioactivity in the tissue at the sample collection time (fractional release, %). The data presented in the manuscript were processed the following way: (1) as a first step, fractional release values in every time point were converted to percentage of baseline i.e. as a percentage of the value found in the sample collected immediately before the OGD was applied (% of baseline). (2) As a second step the net release evoked by OGD was calculated by the area-under-the-curve (AUC) method, i.e. subtracting the resting release calculated during the prestimulation period, from the release measured during OGD. (3) When the effect of drugs was compared under different conditions these net OGD-evoked Glu release values were expressed as a percentage of control net OGD-evoked Glu release values obtained under identical conditions but in the absence of the given drug (% of control).

The tissue tritium uptake was determined as the sum release + the tissue content after the experiment and expressed in Bq/g. In some experiments the endogenous glutamate level has also been determined in the samples by HPLC with o-phthal dialdehyde derivatization and fluorometric detection according to the method described earlier (Nakai et al., 1999) and expressed in pmol/ml.

Determination of adenine nucleotides and energy charge (EC)

Tissue adenine nucleotides were measured at the end of the release experiment by reversed phase high pressure liquid chromatography combined with ultraviolet detection (HPLC-UV), according to the modified method of Milusheva et al. (1996) and described earlier (Sperlágh et al., 2003), using gradient elution. A Gilson liquid chromatographic system was used for the analysis. Separation of adenine nucleotide was performed on 5 μm Discovery C18 (150 \times 4.6 mm; Supelco, Bellefonte, PA, USA) analytical column at ambient temperature. The mobile phase A was 0.075 M potassium phosphate buffer pH 5.5, the B component methanol and acetonitrile in 3.5:1; the gradient profile was linear to 20.8% (V/V) of B at 45 min time of analysis. The flow rate at the beginning of separation was 0.6 ml/min and increased to 1.0 ml/min from 18 min to 38 min of analysis time, linearly. The effluent was monitored with a UV detector (ABS 785/A; Applied Biosystems, Foster City, CA, USA) at 254 nm wavelength. At the end of the release experiment the tissue slices were immediately frozen in liquid nitrogen. Then 20 mg of tissue was homogenized in 200 μl 1.0 M perchloric acid that contained theophylline (as an internal standard) at 10 μM concentration. The suspension was centrifuged at 3500 $\times g$ for 10 min at 4 $^{\circ}\text{C}$. The perchloric anion was precipitated by the addition of 15 μl of 10 M KOH to 185 μl of supernatant. The precipitate was then removed by centrifugation. The supernatant was kept at -20°C until analysis. The pellet was saved for protein measuring according to Lowry et al. (1951).

The identification of different purines was based on the retention times of known amount of standards and was carried out by the Agilent ChemStation program (Agilent Technologies, Wilmington, DE, USA). The amount of nucleotides was quantified from the peak area in the chromatogram with the standard addition methods. A linear correlation between the peak area and the injected amount was observed for all of the nucleotides. Adenylate EC was calculated as described by Atkinson (1968):

$$\text{EC} = \frac{[\text{ATP}] + 0.5[\text{ADP}]}{[\text{ATP}] + [\text{ADP}] + [\text{AMP}]}$$

When the decomposition of exogenous ATP in the extracellular fluid was measured, hippocampal slices were incubated in

3 ml of Krebs solution at 37 $^{\circ}\text{C}$, bubbled with 95% O_2 + 5% CO_2 in the absence or presence of ARL67156 (50 μM). Subsequently ATP (20 μM) was added to the bath and aliquots of 70 μl were taken out at 2.5, 5, 10, 15, 20, 25, 30 and 60 min after the addition of ATP. The concentrations of ATP in the aliquots were measured by HPLC-UV as described above. The actual concentrations of ATP were expressed in μM .

Preparation of rat hippocampal membranes

Wistar male rats (170–190 g) were decapitated and the brain was kept in ice cold Krebs solution oxygenated with 95% O_2 + 5% CO_2 . Both hippocampi were dissected out, weighted, sliced (400 μm) and perfused in 1 ml Krebs solution at a rate of 0.6 ml/min for 30 min at 37 $^{\circ}\text{C}$ with continuous oxygenation. Then the slices were exposed to ischemic-like condition (OGD) by perfusing them for 48 min with Krebs solution saturated with 95% N_2 and 5% CO_2 lacking glucose, whereas control slices were perfused with normal Krebs solution oxygenated with 95% O_2 and 5% CO_2 . Then the tubes containing the slices were cooled in ice bath and Krebs solution was removed. Slices were homogenized in 0.32 M sucrose (1:10 g/ml) using a Teflon–glass homogenizer (Wheaton, USA). The nuclei were removed by centrifugation at 1000 $\times g$ for 10 min and the supernatant was further centrifuged at 40,000 $\times g$ for 15 min. The pellet was suspended by ultra turrax in 50 mM Tris–HCl (pH 7.4) containing 2 mM EGTA, 1 mM EDTA, 5 U/ml adenosine deaminase (ADA) and incubated for 30 min at 37 $^{\circ}\text{C}$ to remove endogenous adenosine. After centrifugation at 40,000 $\times g$ for 15 min the pellet was washed and centrifuged again and taken up in 1:30 (g/ml) 50 mM Tris–HCl (pH 7.4) buffer containing 10 mM MgCl_2 . Membrane preparation was not stored, it was used immediately for binding experiments.

^3H -2-(4-(2-p-carboxyethyl)phenylamino)-5,N-ethylcarbox-amidoadenosine (^3H -CGS21680) binding

An aliquot of membrane preparation was incubated for 3 h at 25 $^{\circ}\text{C}$ with different concentration of ^3H -CGS21680 (5 nM–200 nM) in a final volume of 250 μl Tris–HCl buffer (pH 7.4) containing 10 mM MgCl_2 and 5 U/ml ADA. To study the “typical” and “atypical” sites of ^3H -CGS21680 binding (Cunha et al., 1996) experiments were performed in the presence of 20 nM DPCPX or 200 nM 8-(3-chlorostyryl)-caffeine (CSC) respectively. The incubation was initiated by adding the tritium-labeled ligand and was terminated by rapid vacuum filtration (Brandel cell harvester, Gaithersburg, MD, USA) over GF/B Whatman filters soaked in 0.05% polyethyleneimine. The radioactivity trapped on the filters was counted in Ultima Gold scintillation cocktail (Packard) in a Packard Tricarb 1900 counter for 10 min. Non-specific binding was defined by using the $\text{A}_1/\text{A}_2\text{A}$ antagonist 9-chloro-2-(2-furanyl)-(1,2,4)triazolo(1,5-c) quinazolin-5 amine (CGS15943) in 10 μM concentration. Each concentration of the ligand was in triplicate. The protein concentration of the assay was 0.2–0.3 mg/ml, the protein content was measured by modified method of Lowry et al. (1951) using CuEDTA. The dissociation constant (K_d) and the receptor density (B_{max}) were calculated by the Prism 3 program (San Diego, CA, USA).

Materials

L-[G- ^3H]glutamate (spec. act. 49 Ci/mmol) was obtained from Amersham (Little Chalfont, Buckinghamshire, UK), [^3H]CGS21680 (spec. act. 40.5 Ci/mmol) was purchased from New England Nuclear (Perkin-Elmer, Waltham, MA, USA). The following drugs were obtained from Sigma (St. Louis, MO, USA): ADA, ATP, ADP, AMP, BBG, CSC, PPADS, SCH58261, TTX. CGS15943, CGS21680, DPCPX and ZM241385 were purchased from Tocris (Avonmouth, UK). All other chemicals were from Merck. DPCPX, SCH58261 and ZM241385 for release experiments were dis-

solved in 0.1% DMSO, all the other drugs were dissolved in double-distilled water. The solvent alone did not significantly affect the basal or OGD-evoked [³H]glutamate efflux. For binding studies DPCPX, CSC and CGS15943 were dissolved in DMSO and diluted with incubation buffer to the appropriate concentration. All solutions were freshly prepared on the day of use.

Statistics

All data were expressed as means ± S.E.M. of *n* observations. The statistical analyses were made by one or two-way analysis of variance (ANOVA) followed by Dunnett's post hoc test (multiple comparisons), or Student's *t*-test (pairwise comparisons). *P* values of less than 0.05 were considered statistically significant.

RESULTS

After 60-min preperfusion the radioactivity taken up by the tissue was 107.1 ± 5.7 kBq/g (*n* = 13). The basal tritium efflux measured in a 3-min sample was 3.04 ± 0.09% (*n* = 13) of tissue tritium content, which remained relatively constant during the subsequent sample collections. OGD elicited a stepwise elevation of [³H]glutamate release starting 9 min after the onset of the treatment, and reaching a clearly defined maximum only after 39 min of perfusion (Fig. 1A). The tritium efflux is elevated from 2.66 ± 0.12% to 4.23 ± 0.23% at this point, which represented a 59 ± 5.5% increase over the baseline, and the net release evoked by OGD was 532.82 ± 35.63% (*n* = 27) (Fig. 1A, B). OGD also significantly increased the endogenous glutamate level in the effluent (from 30.52 ± 10.28 to 108.74 ± 27.02 pmol/ml, *n* = 4, *P* < 0.05). Preliminary experiments showed that shorter periods of OGD did not result in a consistent elevation of tritium efflux; therefore it was necessary to apply a rather prolonged treatment, which did not allow the routine detection of the restoration of the response. Indeed, this treatment elicited a massive reduction in the tissue ATP and ADP content, with a simultaneous decrease in the EC (Fig. 1C, D).

Omission of Ca²⁺ from the perfusion solution, whereas it significantly elevated the basal release (5.17 ± 0.61%, *n* = 4, *P* < 0.001), did not change the effect of OGD on [³H]glutamate release (Fig. 1B). In contrast, the sodium channel inhibitor TTX (1 μM) almost fully abolished OGD-evoked [³H]glutamate release (Fig. 1B). These findings indicate that glutamate efflux, evoked by OGD is Ca²⁺ independent, but is dependent on the activity of voltage activated sodium channels. Therefore its most likely underlying mechanism is the sodium-dependent reversal of the glutamate transporters, as described earlier (Rossi et al., 2000). Supporting this assumption, cooling the bath temperature to 12 °C, which inhibits transporter-mediated processes, but leaves unaffected the exocytotic transmitter release (Vizi and Sperl agh, 1999), decreased the resting [³H]glutamate outflow (1.97 ± 0.17%, *n* = 6, *P* < 0.001) and completely inhibited OGD-evoked [³H]glutamate release (Fig. 1B).

In order to reveal the action of endogenous purines, specific antagonists acting at various subtypes of their receptors were utilized. At first we tested their effect under normoxic conditions (Table 1) to reveal any potential confounding interaction on their action on OGD-evoked

[³H]glutamate efflux. Out of the antagonists, only DPCPX (250 nM), the selective A₁ adenosine receptor antagonist affected significantly the resting [³H]glutamate efflux from the slices: it enhanced the tritium level in the effluent, indicating that endogenous adenosine acts tonically to diminish basal glutamate release in the hippocampus. On the other hand ZM241385 (20 nM), SCH58261 (100 nM), the selective A_{2A} receptor antagonists, PPADS (10 μM), the P2 receptor antagonist with wide subtype specificity, BBG (100 nM), the selective P2X7 receptor antagonist had no significant effects on tritium efflux at rest, showing that basal tritium efflux is not subject to neuromodulation by the above receptors under normoxic-normoglycemic conditions (Table 1). When ATP (1 mM) was added to the perfusion solution, it increased basal [³H]glutamate efflux, and its effect was attenuated by PPADS (10 μM), the P2 receptor antagonist. Similarly, when adenosine (1–10 μM) was added, it also elevated tritium efflux and this effect was antagonized by ZM241385 (20 nM) (Table 1). These observations show that facilitatory P2 and A_{2A} receptors, although not activated by endogenous ATP, are functional under normoxic conditions, and exogenously added adenosine in this concentration range preferentially activates A_{2A} receptors, whereas A₁ receptors are already activated by endogenous adenosine. Since it has been already clarified in previous studies (Corradetti et al., 1984; Burke and Nadler, 1988; Sehmisch et al., 2001; Lopes et al., 2002), we did not test the effect of these drugs on depolarization-evoked release of [³H]glutamate here. Instead, the above experiments were designed to reveal any interaction of these drugs on basal efflux, which could interact with their action under energy deprivation.

Next, we examined whether all these antagonists affect increased [³H]glutamate efflux evoked by OGD. Antagonists were perfused from 30 min before the beginning of the collection period, i.e. about 42 min before the onset of ischemic-like conditions. PPADS, the P2 receptor antagonist with wide subtype selectivity, was applied in the concentration range of 0.1–10 μM, in which it does not affect glutamatergic currents or excitatory postsynaptic currents and ecto-ATPase activity as well (Khakh et al., 2003). In this range PPADS concentration-dependently inhibited OGD evoked [³H]glutamate efflux, although the maximal effect was obtained already at 1 μM (Fig. 2A, B).

BBG, the selective P2X7 receptor antagonist also concentration-dependently attenuated OGD-evoked [³H]glutamate release in the P2X7 receptor selective concentration range (0.1–100 nM) (net OGD evoked [³H]glutamate efflux: 275.2 ± 26.6% at 100 nM, 51.64 ± 4.99% of control *n* = 11, *P* < 0.05, Fig. 2A, B). Because BBG then also acts at other P2X receptors, higher concentrations were not tested.

Interestingly, DPCPX (0.1–250 nM), the selective A₁ adenosine receptor antagonist also decreased OGD-evoked [³H]glutamate efflux under our experimental conditions (Fig. 3A, B). On the other hand, ZM241385 (0.1–20 nM), the selective A_{2A} receptor antagonist was the most potent compound to inhibit the outflow of [³H]glutamate

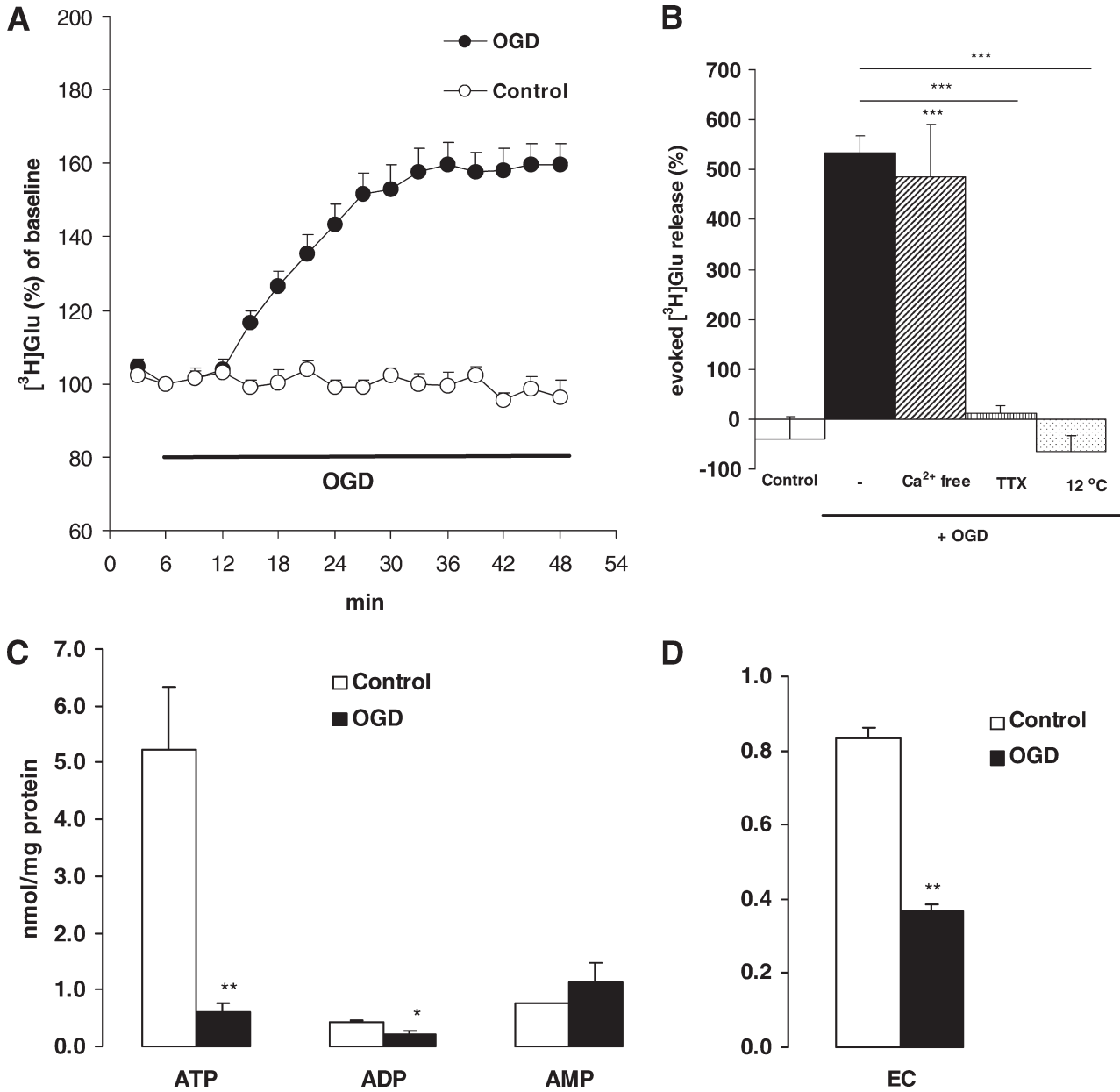


Fig. 1. OGD increase [3H]glutamate efflux in superfused rat hippocampal slices (A) by sodium channel activation and subsequent reversal of the glutamate transporters (B), depletes tissue ATP stores (C), which results in energy deprivation (D). (A) Hippocampal slices were preloaded with [3H]glutamate and then superfused. After a 60-min preperfusion, perfusate samples were collected and the slices were exposed to OGD by the omission of the glucose and the replacement of 95% O₂+5% CO₂ with 95% N₂+5% CO₂ from the perfusion solution according to the horizontal bar. The results of these experiments are illustrated by filled circles, whereas in control experiments (open circles) identical solution was changed for the same period. The release of glutamate is expressed as a percentage of baseline (for calculation see Experimental Procedures). The curves show the mean±S.E.M. of 13–27 experiments. (B) The effect of Ca²⁺-free conditions (Ca²⁺ free), TTX (1  M) and low temperature (12  C) on OGD-evoked [3H]glutamate efflux from rat hippocampal slices. For details of different treatments, see Experimental Procedures. The release of [3H]glutamate evoked by OGD was calculated by the AUC method, i.e. subtracting the resting release, calculated from the prestimulation period, from the release measured in the presence of OGD treatment. Asterisks represent significant differences from controls or as indicated by horizontal bars (*n*=8–16, *** *P*<0.001). (C) The effect of OGD on tissue content of ATP, ADP and AMP under identical treatment illustrated in A. Tissue adenine nucleotides were measured at the end of release experiment by HPLC-UV. Adenine nucleotide content was expressed in nmol/mg protein. Asterisks indicate significant differences from controls (*n*=4, * *P*<0.05, ** *P*<0.01). (D) The effect of OGD on tissue EC under identical treatment illustrated in A. EC was calculated according to Atkinson (1968), for details see Experimental Procedures. Asterisks indicate significant differences from controls (*n*=4, ** *P*<0.01).

evoked by OGD eliciting about 81% reduction at 20 nM concentration; and this effect was again concentration-dependent within the A_{2A}-receptor selective 0.1–20 nM

range (Fig. 3A, B). When DPCPX (250 nM) and ZM241385 (20 nM) were added together, no further inhibition of OGD-evoked [3H]glutamate efflux was observed: the net release

Table 1. Effect of purinergic drugs on the resting efflux of [³H]glutamate from rat hippocampal slices under normoxic conditions

Drug	Resting [³ H]glutamate efflux (%)
–	2.95±0.06
PPADS, 10 μM	3.28±0.20
Brilliant Blue G, 100 nM	3.05±0.29
DPCPX, 250 nM	3.74±0.20*
ZM241385, 20 nM	2.71±0.12
SCH58261, 100 nM	2.74±0.05
ARL67156, 50 μM	3.24±0.19
ATP, 1 mM	4.10±0.12**
ATP, 1 mM+PPADS, 10 μM	3.37±0.15
Adenosine, 1 μM	3.17±0.16
10 μM	3.74±0.19*
Adenosine 10 μM+ZM241385 20 nM	2.71±0.12

Resting [³H]glutamate efflux was calculated by taking the average of the fractional release found in four consecutive samples immediately after drug addition, and the time analogous samples were taken into account in case of drug-free control experiments. Results were analyzed by one-way ANOVA followed by the Dunnett test ($n=4-8$).

* $P<0.05$, statistically significant differences from drug-free controls.

** $P<0.01$, statistically significant differences from drug-free controls.

evoked by OGD was 159 ± 38 , 105 ± 35 and $234\pm29\%$ in the presence of DPCPX, ZM241385 and ZM241385+DPCPX, respectively, not significantly different from each other ($n=8-12$, $P>0.05$). SCH58261 (2–100 nM) another selective A_{2A} receptor antagonist also significantly decreased OGD-evoked [³H]glutamate release (net OGD evoked release: $324.1\pm63.3\%$, at 100 nM, $60.8\pm10.7\%$ of control, $n=8$, $P<0.05$), but with less potency than either DPCPX or ZM241385 (Fig. 3B).

Under normoxic conditions and short-term ischemia the endogenous activation of A_1 receptors decreases glutamate efflux and therefore inhibition of A_1 receptors by specific antagonists elicits an increase in glutamate efflux (Table 1, Corradetti et al., 1984; Marcoli et al., 2003). On the contrary, the endogenous activation of A_{2A} receptors enhances glutamate release, and therefore pharmacological antagonism of A_{2A} receptors results in the attenuation of glutamate efflux (Marcoli et al., 2003). However, it was shown previously that in rat hippocampus most of the binding of A_{2A} receptor agonist CGS21680 was displaced by the A_1 receptor antagonist DPCPX at low nanomolar concentration and two high affinity (typical and atypical) binding sites of CGS21680 were detected using different adenosine receptor agonists and antagonists (Cunha et al., 1996b). Therefore, to examine the effect of long-term OGD on the dissociation constant and receptor density of the two distinct sites of adenosine A_{2A} receptor, and thereby shed light on the paradoxical, inhibitory effect of DPCPX on OGD-evoked [³H]glutamate efflux, ³H-CGS21680 binding experiments were performed in membranes of rat hippocampal slices. The binding of ³H-CGS21680 was saturable and reversible. After 3 h of incubation at 25 °C the specific binding reached the maximum value. To study the binding to the typical site we covered with 20 nM DPCPX that site of the adenosine receptor, which show high affinity for this A_1 receptor an-

tagonist. The binding of ³H-CGS21680 to the typical site in control slices (no OGD) had a K_d of 25.3 ± 5.8 nM and B_{max} of 60.1 ± 8.9 fmol/mg protein ($n=4$). After ischemic condition no significant change was found, the K_d was 27.9 ± 6.2 nM, and the B_{max} was 70.9 ± 15.6 fmol/mg protein ($n=4$). It was reported (Jacobson et al., 1993) that CSC has high affinity for striatal A_{2A} receptors as an antagonist, therefore we used it in 200 nM concentration to cover the typical (striatal like) binding site and measure the binding to the atypical site. The K_d of this site in control slices (no OGD) was 70.8 ± 10.2 nM and the receptor density (B_{max}) was equal with 349.5 ± 21.7 fmol/mg protein. Fig. 4 shows the saturation curves of the atypical site of ³H-CGS21680 binding in control hippocampal slices in comparison with slices undergone to ischemia. It can be seen that ischemia did not change significantly either the affinity of the receptor site or the B_{max} value. After ischemia the K_d was 57.1 ± 8.4 nM and the B_{max} 340 ± 28.4 fmol/mg protein.

These findings indicated that endogenous purines released upon energy deprivation facilitate [³H]glutamate efflux, via activation of both P2 and adenosine receptors. As an alternative approach to influence the level of endogenous purines at the vicinity of receptors, the specific ecto-ATPase inhibitor ARL67156 was utilized. To test whether ARL67156 is indeed able to inhibit the extracellular metabolism of ATP, exogenous ATP was added to hippocampal slices and its degradation was determined by the HPLC-UV technique. In the presence of ARL67156 (50 μM), the hydrolysis of ATP (20 μM) significantly slowed down (Fig. 5A), but was not completely stopped, and similar results were obtained at higher initial ATP concentrations (data not shown).

When applied under normoxic conditions, ARL 67156 (50 μM) did not significantly change the resting efflux of [³H]glutamate (Table 1). When preperfused 42 min before the onset of OGD, the amount of tritium released by OGD was slightly, but insignificantly less in the presence of ARL67156 (net OGD-evoked [³H]glutamate efflux: $415\pm36\%$ of the baseline, $n=4$, $P>0.05$ vs. OGD alone). When ARL67156 was perfused together with P2 receptor antagonists, their inhibitory effects were potentiated by the inhibition of extracellular breakdown of ATP (Fig. 5B). The action of BBG (100 nM) was strongly potentiated by ARL67156 and no net increase in [³H]glutamate efflux in response to OGD was detected under these conditions; instead a net decrease was observed (net OGD-evoked [³H]glutamate efflux: $-116\pm44\%$ of the baseline, $-0.27\pm0.10\%$ of ARL alone, $n=8$, Fig. 5B). In contrast, when the decomposition of endogenous ATP was slowed down by ARL67156, the inhibitory effect of both DPCPX and ZM241385 was virtually abolished (Fig. 5B).

DISCUSSION

This study was designed to explore the participation of endogenously released purines and their action on different nucleotide and adenosine receptors in the modulation of excess glutamate efflux upon a prolonged energy de-

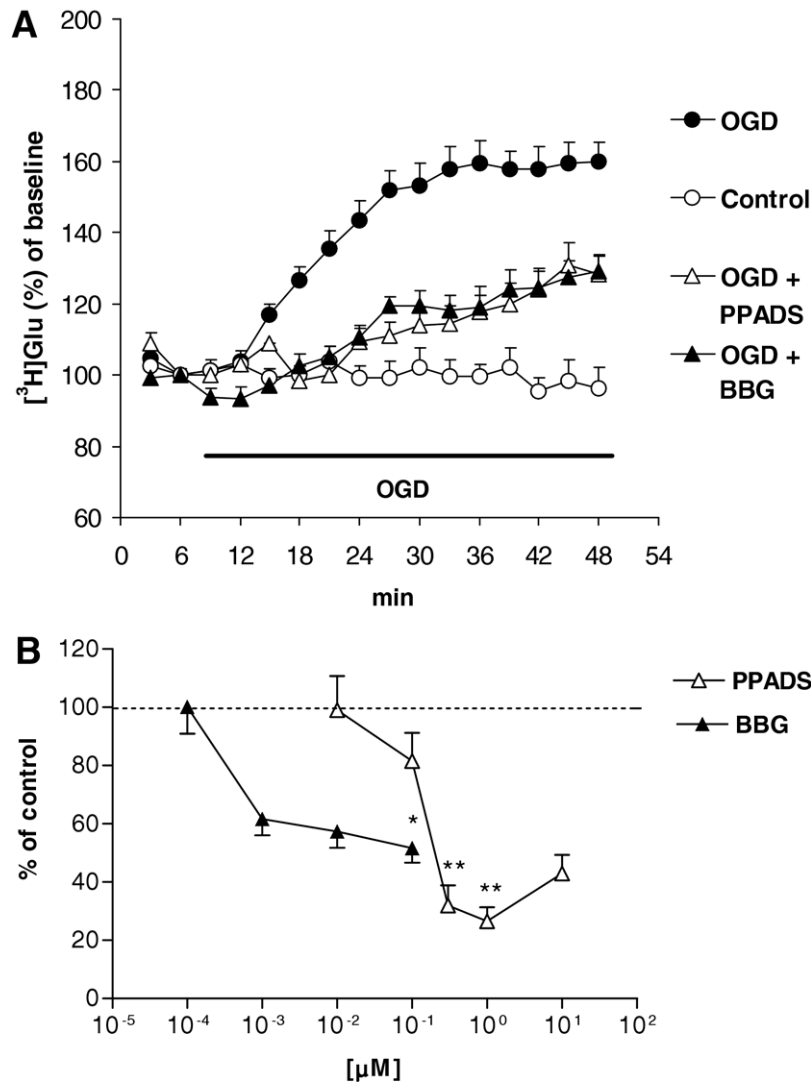


Fig. 2. Effect of P2 receptor antagonists on OGD-evoked [³H]glutamate efflux from rat hippocampal slices. (A) Hippocampal slices were exposed to OGD, as indicated by the horizontal bar in the absence and presence of the P2 receptor antagonist PPADS (10 μM) or the selective P2X7 receptor antagonist BBG (BBG, 100 nM). The perfusion with antagonists started 30 min before the beginning of the sample collection period. The release of glutamate is expressed as a percentage of baseline (for calculation see Experimental Procedures). (B) Concentration-dependence of the inhibitory effect of PPADS and BBG on OGD-evoked [³H]glutamate efflux. The experiments were performed according to the protocol shown in A, with different concentrations of P2 receptor antagonists. The release of [³H]glutamate evoked by OGD was calculated by the AUC method, i.e. subtracting the resting release calculated from the prestimulation period, from the release measured in the presence of OGD treatment and is expressed as a percentage of drug-free controls. The data represent the means ± S.E.M. of 6 to 27 experiments.

privation elicited by *in vitro* ischemia. Our findings suggest that under our experimental conditions, the elevation of $[Na^+]_i$ by the insufficient energy supply, the resultant activation of voltage sensitive sodium channels (VSSC), and the subsequent reversal of sodium-dependent glutamate transporters due to intracellular Na^+ overload might be responsible for the initiation of glutamate efflux, as it was completely $[Ca^{2+}]_o$ independent, but temperature-dependent and sensitive to the blockade of VSSCs by TTX. This is consistent with the mechanism reported by other investigators using rat hippocampal slices (Roettger and Lipton, 1996; Rossi et al., 2000), and *in vivo* blood vessel occlusion models (Seki et al., 1999; Phillis et al., 2000) using relatively long ischemic episodes.

The principal new finding of the present study is the attenuation of long-term OGD-evoked [³H]glutamate efflux by P2 receptor antagonists including PPADS, the P2 receptor antagonist with wide subtype selectivity and the P2X7 receptor selective antagonist BBG. These observations indicate that ATP released from the hippocampus upon energy deprivation activates various subtypes of P2X receptors to elicit glutamate release, which then contributes to excess glutamate overflow from the slices. Although OGD-evoked [³H]glutamate efflux persisted in the absence of extracellular Ca^{2+} , P2X receptors are non-selective cation channels. Therefore, a potential underlying mechanism of excess glutamate release could be direct sodium influx through the receptor ion channel com-

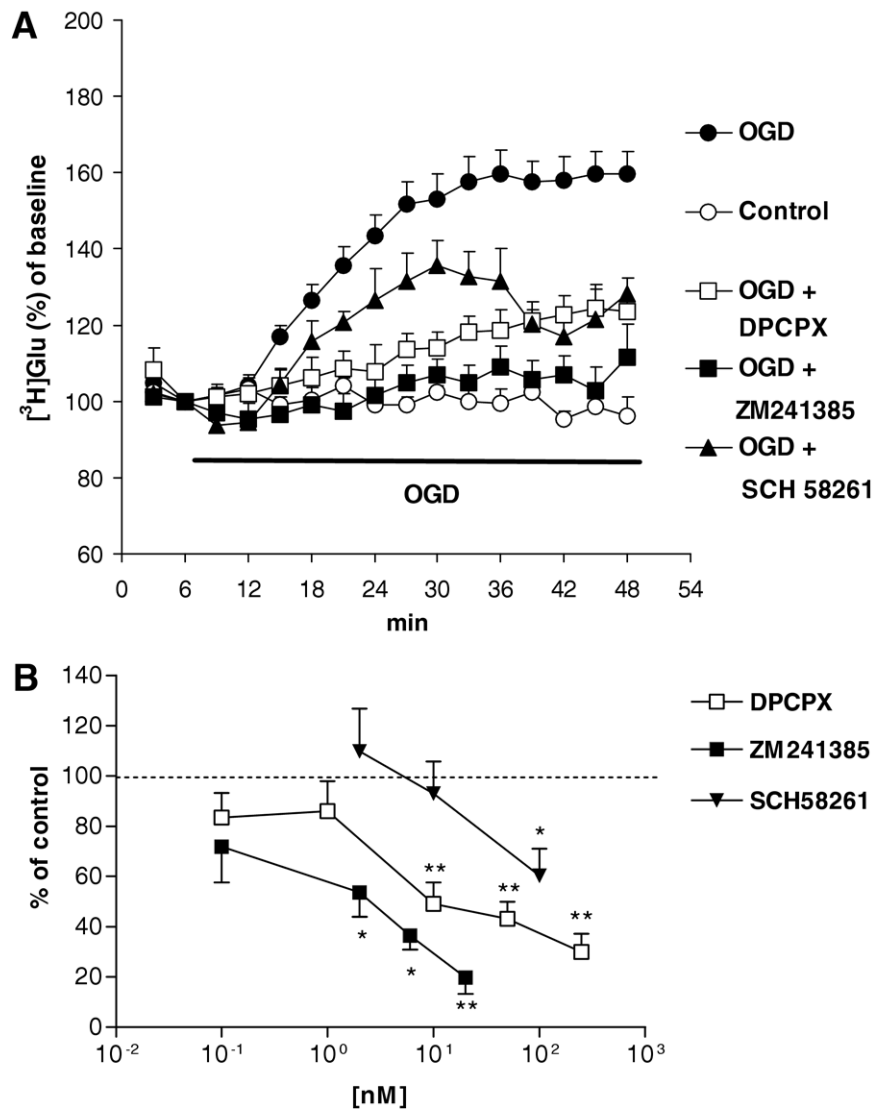


Fig. 3. Effect of adenosine receptor antagonists on OGD-evoked [³H]glutamate efflux from rat hippocampal slices. (A) Hippocampal slices were exposed to OGD, as indicated by the horizontal bar in the absence and presence of the A₁ receptor antagonist DPCPX (250 nM) or the selective A_{2A} receptor antagonists ZM241385 (20 nM) and SCH58261 (100 nM). The perfusion with antagonists started 30 min before the beginning of the sample collection period. The release of glutamate is expressed as a percentage of baseline (for calculation see Experimental Procedures). (B) Concentration-dependence of the inhibitory effect of DPCPX, ZM241385 and SCH58261 on OGD-evoked [³H]glutamate efflux. The experiments were performed according to the protocol shown in A, with different concentrations of adenosine receptor antagonists. The release of [³H]glutamate evoked by OGD was calculated by the AUC method, i.e. subtracting the resting release, calculated from the prestimulation period, from the release measured in the presence of OGD treatment and is expressed as a percentage of drug-free controls. The data represent the means ± S.E.M. of 6 to 27 identical experiments.

plex. The sodium influx could contribute to excess sodium load caused by the energy depletion and the resultant depolarization could trigger voltage-sensitive Na⁺ channels and lead to the reversal of the glutamate uptake emptying cytoplasmic glutamate storage. Because we investigated the antagonism of endogenous agonists acting on P2 receptors, the pharmacological profile of the participating receptor subtypes is necessarily incomplete. Therefore, as for the involvement of individual subtypes of P2 receptors, only some cautious assumptions can be made. Thus, although PPADS could also act on certain subtypes of P2Y receptors, their involvement in this effect

is not very likely, because the activation of P2Y receptors inhibits the efflux of [³H]glutamate in the hippocampus under normoxic/normoglycemic conditions (Rodrigues et al., 2005). The hippocampus expresses mRNA encoding all known subunits of the P2X receptor family (Papp et al., 2004a). Among P2X receptors of known pharmacological profile, P2X1, P2X2, P2X3, P2X5, P2X2/3, P2X4/6, and P2X7 are sensitive to PPADS at the low micromolar range (Le et al., 1998; North and Surprenant, 2000), whereas at the nanomolar concentration range, BBG is selective for P2X7 receptors (Jiang et al., 2000). Therefore the activation of any of these homo- or heterooligomeric assemblies

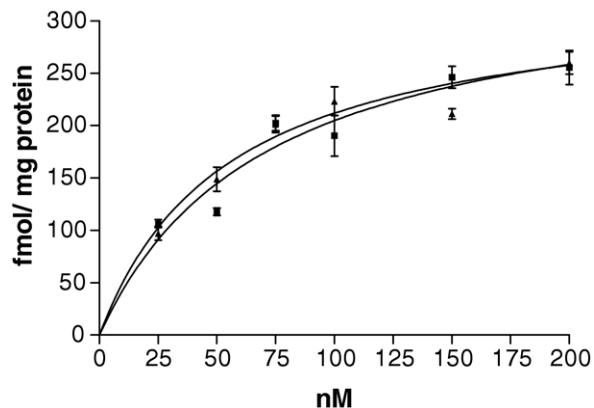


Fig. 4. Saturation curve of ^3H -CGS21680 binding (atypical site) in rat hippocampal control (no OGD, triangle) and ischemic (OGD, square) slices. The ordinate represents the specific binding obtained by the subtraction of non-specific binding from the total binding, the non-specific binding was determined by using $10\ \mu\text{M}$ CGS 15943. Close to the K_d value the specific binding was 25% of the total and was in agreement with previously published data. Curve was generated by using Prism 3 program, results are means \pm S.E.M., number of independent experiments was four, each concentration was in triplicate.

might be responsible for the facilitation of glutamate efflux. However, due to the switch of the metabolic routes to anaerobic pathways, the pH is expected to fall upon hypoxic–hypoglycemic conditions, which prefer the activation of certain subtypes of P2X receptors, i.e. P2X2 and P2X2/3, whereas others, such as P2X1 and P2X3 decrease their activity at lower pH (North and Surprenant, 2000). Moreover the up-regulation of P2X2, P2X4 and P2X7 receptors in hippocampal (Cavaliere et al., 2003, 2004) and cortical (Franke et al., 2004) neurons under *in vitro* and *in vivo* ischemia-like conditions renders these subtypes as likely candidates for the contribution to OGD-evoked ^3H glutamate efflux under our experimental conditions.

As a consequence of persistent facilitation of glutamate release, endogenous overactivation of a certain set of P2X receptors could participate in OGD-evoked neuronal injury as an amplifying factor of glutamatergic excitotoxicity. Concordant with this idea, PPADS inhibited increased glutamate release from excitatory terminals synapsing onto medium spiny neurons in the striatum with a presynaptic mode of action after transient forebrain ischemia (Zhang et al., 2006). P2 receptor antagonists exhibit neuroprotective action in *in vitro* models of neurotoxicity induced by hypoxia–hypoglycemia in cerebellar granule neurons (Cavaliere et al., 2001, 2002); in organotypic hippocampal slice culture (Cavaliere et al., 2003, 2004; Runden-Pran et al., 2005) and in *in vivo* models of ischemia (Kharlamov et al., 2002; Franke et al., 2006; Melani et al., 2006) although P2X7 receptor knockout mice do not show resistance in the MCAo model (Le Feuvre et al., 2003). It is important to stress, however, that in order to evaluate the *in vivo* neuroprotection by different P2 receptor antagonists one should also consider that they affect various other aspects of neurodegeneration and the following repair process, including microglial and astroglial

activation, which could also modify their potential (Volonte et al., 2003; Franke and Illes, 2006; Franke et al., 2006; Sperlagh et al., 2006).

The neuroprotective role of adenosine receptor ligands is also well documented, although still controversial (Ribeiro et al., 2002). Our results extend previous observations that OGD-evoked glutamate release is subject to modulation by facilitatory A_{2A} receptors in the rat (O'Regan et al., 1992; Marcoli et al., 2003) and human (Marcoli et al., 2004) cerebral cortex. However, in the study of Marcoli et al. (2003), A_{2A} receptor antagonists did not affect ischemia-evoked glutamate release under Ca^{2+} free conditions, which implicates that A_{2A} receptors only regulate Ca^{2+} dependent glutamate release upon ischemia. Although our observation do not exclude the possibility that $[\text{Ca}^{2+}]_o$ -dependent glutamate release is subject to facilitation via A_{2A} receptors, in our experiments the OGD-evoked ^3H glutamate efflux was completely $[\text{Ca}^{2+}]_o$ -independent, but still sensitive to modulation by A_{2A} antagonists. A potential explanation to this discrepancy that in the study of Marcoli et al. (2003), a shorter period of oxygen–glucose deprivation was used, which indicates that the duration of ischemia critically determines the activation pattern of different purine receptors involved in the regulation of glutamate release.

Moreover, the longer period of OGD might also serve as an explanation to the finding that DPCPX, the selective A_1 -adenosine receptor antagonist in our hands decreased ischemia-evoked ^3H glutamate efflux instead of facilitation, which would be expected if the A_1 receptor-mediated inhibitory influence of endogenous adenosine on glutamate release is relieved. The lack of a facilitatory effect of DPCPX might be explained by the desensitization of A_1 adenosine receptors upon long-term energy deprivation. Indeed, in other studies, where DPCPX prevents ischemia-induced synaptic depression caused by A_1 -receptor mediated endogenous inhibition of glutamate release (Latini et al., 1999a,b), shorter periods of ischemic-like conditions were applied. Consistently with this assumption Coelho et al. (2006) have showed that the functional responsiveness of A_1 adenosine receptors is decreased upon a prolonged (60 min) OGD, which might be due to endogenous agonist-induced internalization of A_1 receptors (Coelho et al., 2006). This might be also promoted by the simultaneous activation of A_{2A} receptors which has been described to mediate the heterologous desensitization of A_1 receptors in the striatum (Dixon et al., 1997). A_1 and A_{2A} adenosine receptors are coexpressed in hippocampal nerve terminals (Cunha et al., 1994) and several studies showed that there is a crosstalk between them (Ribeiro, 1999). These interactions suggest that A_1 receptor-mediated actions of endogenous adenosine may be attenuated if there is a concomitant activation of A_{2A} receptors.

More unexpected is the finding that DPCPX in fact decreased the OGD-evoked glutamate release applied either in purely A_1 -selective lower (0.1–50 nM) or higher (250 nM) concentration. One possible explanation is that instead of binding to A_1 receptors which became desensitized, DPCPX acted on other receptor sites in the hip-

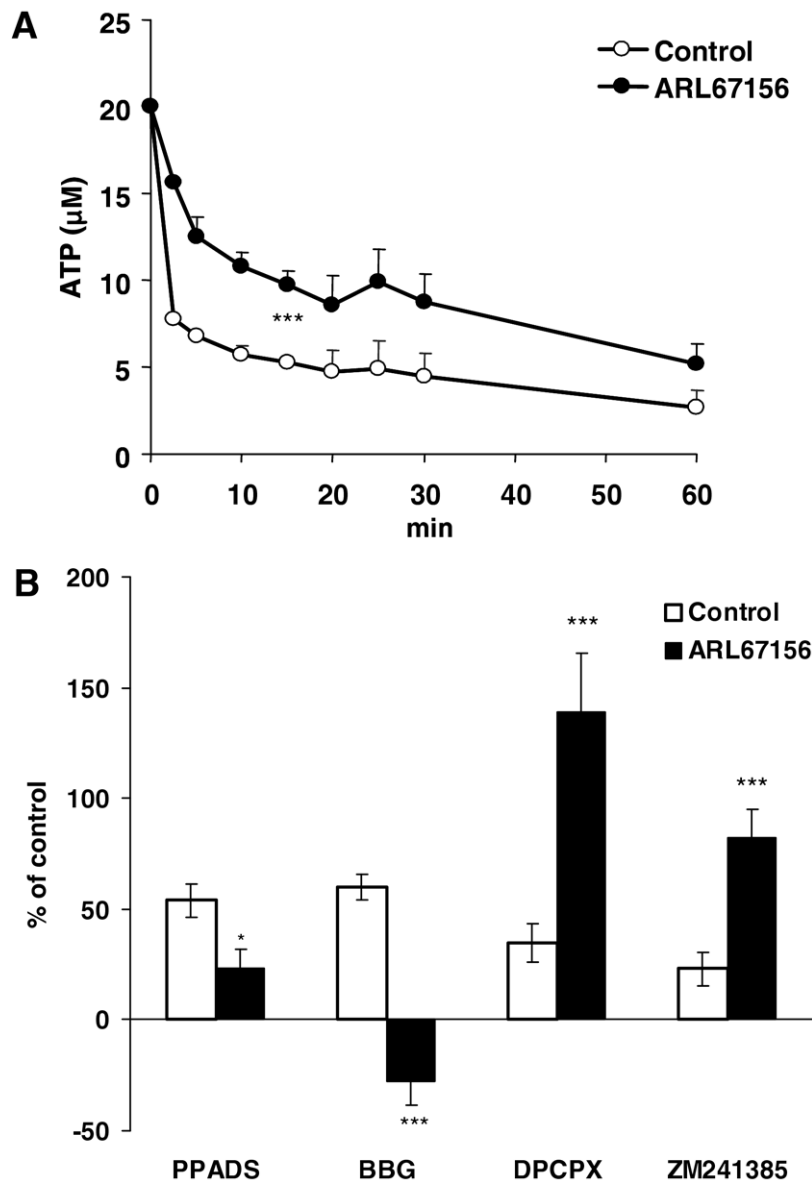


Fig. 5. The ecto-ATPase inhibitor ARL67156 (50 μ M) inhibits the decomposition of extracellular ATP (A) and differentially affects the inhibitory action of P2 and adenosine receptor antagonists on OGD-evoked [3 H]glutamate efflux (B). (A) Rat hippocampal slices were incubated in the presence of 20 μ M ATP, and its amount in the tissue supernatant was analyzed by HPLC-UV, as a function of time, in the absence (open circles) and presence (filled circles) of ARL 67156. The actual concentration of ATP is expressed in μ M. The data represent the means \pm S.E.M. of 3 identical experiments from each series. Asterisks indicate significant difference between ARL67156-treated and control curve calculated by two-way ANOVA (***) $P < 0.001$, which compares two interdependent data sets. Individual data points were also analyzed statistically by unpaired t -test and values at 2.5 min ($P < 0.001$), 5, 10 and 15 min ($P < 0.01$) are significantly different from each other. (B) Hippocampal slices were preperfused with P2 receptor antagonists (PPADS, 10 μ M, BBG, 100 nM) or adenosine receptor antagonists (DPCPX, 250 nM, ZM241385, 20 nM) 30 min before the beginning of the sample collection period, and then were exposed to OGD, according to the protocol shown in Fig. 1A, either in the presence (filled bars) or absence (open bars) of ARL67156 (50 μ M). The OGD-evoked [3 H]glutamate efflux was measured and calculated as described in the Experimental Procedures. In order to compare their action in the absence and presence of ARL67156 the effect of individual drugs was expressed as the percentage of respective controls, i.e. as a percentage of completely drug-free controls in the case of Control and as a percentage of ARL67156-treated, but otherwise drug-free controls in the case of ARL67156. The data represent the means \pm S.E.M. of 8 to 27 identical experiments. Asterisks indicate significant differences between ARL67156-treated and control slices (* $P < 0.05$, *** $P < 0.01$).

pocampus and prevented the action of endogenous adenosine on it. Thus, previous studies indicated, that the prototypical A_{2A} receptor agonist CGS21680 binds to two pharmacologically different binding sites in the rat hippocampus: a typical (striatal like) binding site, which displays pharmacological characteristics of the recombi-

nant A_{2A} adenosine receptor, and is displaced by DPCPX only in the high, submicromolar concentration range and an atypical binding site, which shows high affinity for DPCPX (Johansson and Fredholm, 1995; Cunha et al., 1996b). This atypical binding site, however, is not identical to A_1 -adenosine receptors, as CGS21680 inhibits the bind-

ing of the A_1 radioligand [3 H]CHA (Johansson and Fredholm, 1995; Cunha et al., 1996b), and [3 H]DPCPX (Lopes et al., 2002) only in micromolar concentrations. This binding site comprises the majority (88%) of [3 H]CGS21680 binding sites of the hippocampus and is suggested to mediate the facilitation of synaptic transmission in Schaffer fiber/CA1 pyramid synapses by A_{2A} agonists, an effect reflecting increased glutamate release (Cunha et al., 1996b, 1997). Therefore a plausible explanation to the effect of DPCPX in our experiments is that it bound to the atypical site and thereby prevented the facilitatory effect of endogenous adenosine on glutamate release. Although in more recent studies it was revealed that the atypical binding site of [3 H]CGS21680 is strictly linked to the presence of A_1 adenosine receptors in the cortex and the hippocampus (Halldner et al., 2004; Lopes et al., 2004) it is not identical with the classical A_1 and A_{2A} receptors and therefore these studies interpreted the atypical binding site of [3 H]CGS21680 as a novel binding site or binding mode of A_1 or A_{2A} receptors. To test, whether the atypical binding site is present under ischemic-like conditions we examined the binding of the A_{2A} adenosine receptor ligand [3 H]CGS21680 to hippocampal membranes under normoxic and ischemic condition. In line with previous data [3 H]CGS21680 bound with high affinity to the typical and atypical binding sites, obtained in the presence of DPCPX and CSC, respectively, both under normoxic and ischemic like conditions. There was no significant change in either the K_d , or the B_{max} values of the two distinct binding sites in hippocampal membranes that had undergone long-term ischemic treatment, indicating that neither the affinity, nor the number of the DPCPX sensitive atypical binding site had changed. Moreover SCH58261, which binds only to the typical A_{2A} site (Lindstrom et al., 1996) exhibited relatively low potency at the inhibition of [3 H]glutamate release evoked by OGD, whereas ZM241385, which is known to bind both to typical and atypical binding sites (Cunha et al., 1997), potently decreased [3 H]glutamate efflux in the low nanomolar range. The observation that the effect of DPCPX and ZM241385 was not additive also supports the notion that they share at least partially a common binding site in the hippocampus.

Nevertheless, an effect, which is detected in the presence of an antagonist of a receptor can be either explained by the action of an endogenous ligand or by the constitutive activity of the given receptor. Therefore, as an alternative approach to reveal the action of endogenous purines on their receptors, the selective ecto-ATPase inhibitor ARL67156 was utilized. In fact, ARL67156 significantly slowed down, although did not completely prevent the breakdown of exogenously added ATP, which is in good agreement with the recent observation of Frenguelli et al. (2007). By itself, ARL67156 did not significantly change the amount of [3 H]glutamate efflux evoked by OGD, which indicates that the P2 receptor-mediated endogenous facilitation of [3 H]glutamate efflux by adenine nucleotides keeps a balance with the A_{2A} receptor-mediated facilitation of [3 H]glutamate efflux by endogenous adenosine generated by the extracellular breakdown of ATP. Supporting

this idea, when the effect of antagonists, i.e. the contribution of endogenous agonists to the facilitation of glutamate release was examined in the presence of ARL67156, the effect of P2 receptor antagonists was potentiated, whereas the action of adenosine antagonists was reversed. Thus, in the presence of ARL67156 more endogenous ATP remains to act on P2 receptors, therefore the relief from this facilitation by a P2 receptor antagonist has a stronger effect on glutamate release. Conversely, ARL67156 delays the production of adenosine, which results in a smaller amount of endogenous adenosine at the vicinity of A_{2A} receptors (or at the atypical binding site). Therefore the blockade of the receptors results in a weaker effect, than in the absence of ARL67156.

CONCLUSION

In summary, our data collectively suggest that both P2 and A_{2A} adenosine receptors are endogenously activated under prolonged oxygen and glucose deprivation in the hippocampus: the endogenous activation of both receptors results in the facilitation of glutamate release, whereas inhibitory A_1 adenosine receptors seem to be silent. Therefore antagonists acting at P2 and/or A_{2A} receptors might be the preferable approach to prevent long-term ischemia-evoked glutamate release and thereby attenuate glutamatergic excitotoxicity and resultant neuronal death.

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