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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 [^{3}H]glutamate, which was tetrodotoxin (1 μM)-sensitive and temperature-dependent. The P2 receptor antagonist pyridoxalphosphate-6-azophenyl-2′,4′-disulfonic acid (PPADS, 0.1–10 μM), and the selective $P2X_7$ receptor antagonist Brilliant Blue G (1–100 nM), decreased OGD-evoked [^{3}H]glutamate efflux indicating that endogenous ATP facilitates ischemia-evoked glutamate release. The selective A_1-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 [^{3}H]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 [^{3}H]CGS21680 to hippocampal membranes did not change significantly in response to OGD. Taken together these data suggest that while A_1 receptors might became 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 A_1 adenosine receptors could be an effective approach to attenuate glutamatergic excitotoxicity and thereby counteract ischemia-induced neurodegeneration.

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 subcellular, 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 A_1 (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, P2X2/3, P2X3, (Rodrigues et al., 2005), P2X2 (Khakh et al., 2003) and P2X7 (Sperlagh et al., 2002; Fellin et al., 2006) nucleotide receptors. Under normoxic conditions, the activation of A_1 (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-furanyl)-(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, pyridoxalphosphate-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.

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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 A_1 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 A_1 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 [^{3}H]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

[^{3}H]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, KH_2PO_4 1.2, $MgSO_4$ 1.2, $CaCl_2$ 2.5, $NaHCO_3$ 25, glucose 10, pH 7.4), oxygenated with 95% O_2+5 % CO_2. Both hippocampi were rapidly dissected and 400-μm-thick slices were cut transversely with a McIlwain tissue chopper (Bachofer, Reitlingen, Germany) and incubated in 1 ml oxygenated Krebs solution containing 10 μCi L-[G-^{3}H]glutamate (0.2 μM, 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% O_2+5% CO_2-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 [^{3}H]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% N_2+5 % CO_2. 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-)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 Ca^{2+}-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