Neuroscience 138 (2006) 1195–1203

HYPOXIA-INDUCED DESENSITIZATION AND INTERNALIZATION OF ADENOSINE A_1 RECEPTORS IN THE RAT HIPPOCAMPUS

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Abstract—Activation of A_1 adenosine receptors is important for both the neuromodulatory and neuroprotective effects of adenosine. However, short periods of global ischemia decrease A_1 adenosine receptor density in the brain and it is not known if a parallel loss of functional efficiency of A_1 adenosine receptors occurs. We now tested if hypoxia leads to changes in the density and efficiency of A_1 adenosine receptors to inhibit excitatory synaptic transmission in rat hippocampal slices. In control conditions, the adenosine analog 2-chloroadenosine, inhibited field excitatory post-synaptic potentials with an EC_{50} of 0.23 μM. After hypoxia (95% N_2 and 5% CO_2, for 60 min) and reoxygenation (30 min), the EC_{50} increased to 0.73 μM. This EC_{50} shift was prevented by the presence of the A_1 adenosine receptor antagonist 8-phenyltheophyline, but not by the A_{2A}R antagonist 7-(2-phenylethyl)-5-amino-2-(2-furyl)-pyrazolo-[4,3-e]-1,2,4-triazolo[1,5-c] pyrimidine, during the hypoxic period. This decreased efficiency of A_1 adenosine receptors was not paralleled by a global change of A_1 adenosine receptor density or affinity (as evaluated by the binding parameters obtained in nerve terminal membranes). However, the density of biotinylated A_1 adenosine receptors at the plasma membrane of nerve terminals was reduced by 30% upon hypoxia/reoxygenation, in a manner prevented by the A_1 adenosine receptor antagonist 1,3-dipropyl-8-cyclopentylxanthine and mimicked by prolonged (60 min) supra-maximal activation of A_1 adenosine receptors with 2-chloroadenosine (10 μM). These results indicate that hypoxia leads to a rapid (<90 min) homologous desensitization of A_1 adenosine receptor-mediated inhibition of synaptic transmission that is likely due to an internalization of A_1 adenosine receptors in nerve terminals. © 2005 Published by Elsevier Ltd on behalf of IBRO.

Key words: adenosine, A_1 receptor, hypoxia, desensitization, internalization, hippocampus.

Adenosine is a powerful modulator of neuronal function, which mainly decreases the release of excitatory neurotransmitters and neuronal firing through the activation of inhibitory A_1 receptors (A_1Rs) (Dunwiddie and Masino, 2001). Since adenosine is released in particularly high amounts in noxious situations, adenosine is conceived as an important endogenous neuroprotective agent against different noxious insults to the brain (de Mendonça et al., 2000). Adenosine A_1Rs belong to the G protein-coupled receptor family but, unlike most in their family, A_1Rs have a long half-life (e.g. Hettinger et al., 1998) and seem to be resilient to desensitization (Wetherington and Lambert, 2002). In fact, several works suggest that neuronal A_1R desensitization occurs after a prolonged (12–24 h) exposure to exogenously added A_1R agonists *in vitro* (e.g. Abbracchio et al., 1992; Hettinger et al., 1998; Vendite et al., 1998) as well as *in vivo* (e.g. Fernandez et al., 1996; Ruiz et al., 1996). The time course of desensitization of A_1Rs is particularly critical to understand whether adenosine maintains its neuroprotective efficiency in chronic brain insults. In fact, in a kindling model of epilepsy where a long-lasting enhanced release of adenosine is observed (Berman et al., 2000), there is a long-term decrease in the efficiency of A_1Rs agonists that is accompanied by a decreased density of presynaptic A_1Rs in the hippocampus (Rebola et al., 2003a). Likewise, several studies showed that short periods of global ischemia produce long lasting changes in the density of A_1Rs in several brain regions (e.g. Lee et al., 1986; Nagasawa et al., 1994; Onodera et al., 1987). However, it remains to be determined if this ischemia-induced decrease of A_1R density is paralleled by a decrease of A_1R-mediated responses. These studies also focused on the long-term changes of A_1R density rather than concentrating in the post-ischemic period (0–6 h), considered the clinically relevant time window for pharmacological intervention (e.g. Beresford et al., 2003).

We now took advantage of an *in vitro* model of hypoxia-induced depression of synaptic transmission in rat hippocampal slices (Coelho et al., 2000) to test whether the enhanced release of adenosine known to occur in this model (Frenguelli et al., 2003) hampers the ability of A_1Rs to inhibit excitatory synaptic transmission.

EXPERIMENTAL PROCEDURES

Chemicals

2-Chloroadenosine (CADO), N^6-cyclopentyladenosine (CPA), 1,3-dipropyl-8-cyclopentilxanthine (DPCPX) and 8-phenyltheophyline (8-PT) were from Sigma (Sigma-Aldrich Química, Sintra, Portugal), 8-{4-[(2-aminoethyl)amino]carbonylmethyl-oxyphenyl}xanthine (XAC) was from Research Biochemical International (Reagente 5, Oporto, Portugal), ^{3}H-DPCPX (specific activity 109.0 Ci/mmol) was from DuPont NEN (Anagene, Portugal) and rabbit purified IgG anti-adenosine A_1R antibody (1.8 mg/ml) was from Affinity Bioreagents

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Abbreviations: A_1R, A_1 receptor; CADO, 2-chloroadenosine; CPA, N^6-cyclopentyladenosine; DPCPX, 1,3-dipropyl-8-cyclopentylxanthine; fEPSP, field excitatory post-synaptic potentials; SCH 58261, 7-(2-phenylethyl)-5-amino-2-(2-furyl)-pyrazolo-[4,3-e]-1,2,4-triazolo[1,5-c] pyrimidine; XAC, 8-{4-[(2-aminoethyl)amino]carbonylmethyl-oxyphenyl}xanthine; 8-PT, 8-phenyltheophyline.

0306-4522/06$30.00+0.00 © 2005 Published by Elsevier Ltd on behalf of IBRO.
doi:10.1016/j.neuroscience.2005.12.012

(Golden, CO, USA). SCH 58261 (7-(2-phenylethyl)-5-amino-2-(2-furyl)-pyrazolo-[4,3-e]-1,2,4-triazolo[1,5-c] pyrimidine) was a generous gift from Scott Weiss (Vernalis, UK). DPCPX was made up as a 5 mM stock solution in dimethylsulfoxide with 1% NaOH 1 M (v/v), whereas SCH 58261, CPA, XAC and 8-PT were made up as 5 mM stock solutions in dimethylsulfoxide. Aliquots were kept at −20 °C until used. At the maximal concentrations used (<0.005%), dimethylsulfoxide is devoid of effects on synaptic transmission or on hypoxia-induced depression of synaptic transmission. CADO (1 mM) was prepared daily in Krebs solution.

Electrophysiological recordings in hippocampal slices

Male Wistar rats (5–7 weeks) were handled according to EU guidelines (86/609/EEC), with care to minimize the number of animals used and their suffering. The rats were anesthetized under halothane atmosphere before being killed by decapitation. The brain was rapidly removed to ice-cold Krebs solution (in mM: 115 NaCl, 3 KCl, 1.2 KH_2PO_4, 1.2 $MgSO_4$, 2 $CaCl_2$, 25 $NaHCO_3$, 10 glucose, pH 7.4) saturated with 95% O_2 and 5% CO_2. The hippocampus was dissected free and 400 μm slices were cut with a McIlwain tissue chopper. The slices were allowed to recover for at least one hour in Krebs solution continuously bubbled with 95% O_2 and 5% CO_2 at room temperature (23–26 °C) before starting the experimental protocols.

One hippocampal slice was placed in the recording chamber (1 ml capacity) and continuously superfused with Krebs (3 ml/min), at a temperature of 32 (±0.1) °C. Stimulation of the Schaffer fibers was delivered through a bipolar tungsten electrode every 15 s (0.6 Hz). The recording glass micropipette (2–6 MΩ) was placed in the stratum radiatum of CA1 area, and evoked field excitatory post-synaptic potentials (fEPSP) were acquired and stored in a personal computer with the LTP 1.3 software (Anderson and Collingridge, 2001). Concentration-response curves were performed with consecutive applications of increasing concentrations of CADO (0.15–5 μM) present in the superfusing solution. The application of a new concentration was initiated after a stable value of slope and amplitude of the fEPSP was obtained for the previous concentration. Data were not considered if the recovery after washout of the last CADO concentration did not reach 80–100% of initial slope/amplitude values.

Hypoxia was induced for 60 min, by changing the superfusing solution from 95% O_2 and 5% CO_2 saturated (normoxic) to 95% N_2 and 5% CO_2 saturated (hypoxic) Krebs solution. This caused a drop in pO_2 in the recording chamber from 560 mm Hg (normoxia) to 106 mm Hg (hypoxia) (see Coelho et al., 2000), measured with a pO_2 meter (ISO2 from WPI). Recovery from hypoxia was allowed for at least 30 min before starting a CADO concentration-response curve. When using an antagonist (either 8-PT or SCH 58261), it was applied to the superfusion solution 20 min prior to starting hypoxia and until a complete recovery of the fEPSP responses on re-oxygenation was obtained. A further 30 min period was allowed to wash out the antagonist prior to initiating a CADO concentration-response curve. Thus, the effect of hypoxia/reoxygenation was evaluated by comparison of CADO concentration-response curves in the same slice and was also validated by comparison of these two curves when obtained in different slices from the same animal that were either subject to hypoxia/reoxygenation or normoxia during 90 min. We confirmed that the potency of CADO is maintained over the time course of our experiment in the same slice i.e. two consecutive CADO applications did not elicit desensitization of CADO-induced responses (see also data in the Results section). This enabled us to compare the effects of CADO in the same slice in the absence or in the presence of hypoxia. Importantly, and in accordance with previous studies, we found that the potency of CADO is constant in different slices obtained from the same animal (i.e. there is negligible inter-slice variability), allowing us to perform one CADO curve in single slices (when testing the effect of antagonists), greatly reducing the time of the experimental protocol, and therefore the time-dependent decay of synaptic transmission.

Radioligand-binding experiments

Hippocampal slices from three rats (5–7 weeks) were prepared as previously described, transferred to a 3 ml capacity chamber and continuously superfused (3 ml/min) with Krebs solution saturated with 95% O_2 and 5% CO_2 at 32 °C. Two such chambers were used in parallel to mimic control (normoxia) and hypoxia/reoxygenation conditions, as closely as possible to the electrophysiology experimental conditions. After an initial 30 min of stabilization at 32 °C, hypoxia was induced for 60 min (as described above), and the slices were allowed to recover for 30 min in oxygenated Krebs. The pO_2 levels in these chambers during normoxia (75–80% pO_2) or hypoxia (5–10% pO_2) were similar to those measured in the chamber used in electrophysiology experiments.

At the end of the protocol, the contents of each chamber were separately collected into sucrose solution (in mM, 320 sucrose, 1 EDTA, 5 HEPES and 1 mg/ml BSA, pH 7.4) and homogenized in a Potter-Elvehjem with a Teflon piston (four up-and-down strokes) to prepare nerve terminal membranes, as previously described (Lopes et al., 1999a). Briefly, the homogenate was centrifuged at 3000×g for 10 min, the supernatant collected and centrifuged at 14,000×g for 12 min. The pellet was resuspended in 2 ml of a 45% (v/v) Percoll solution made up in a Krebs–HEPES–Ringer solution (in mM: 140 NaCl, 5 KCl, 25 HEPES, 1 EDTA, 10 glucose, pH 7.4). After centrifugation at 14,000×g for 2 min the top layer was removed (synaptosomal fraction) and washed with 1 ml of Krebs–HEPES–Ringer. Previous studies have confirmed that this fraction is essentially devoid of glial contamination (Cunha et al., 1992) and enriched in presynaptic markers (<5% contamination by post-synaptic density markers, see Rodrigues et al., 2005). After centrifugation at 14,000×g for 2 min, the pellet was resuspended in 50 mM Tris with 2 mM $MgCl_2$ pH 7.4 (Tris/Mg solution) to disrupt the nerve terminals. The membranes were then recovered as a pellet following centrifugation at 20,000×g for 20 min.

Binding assays

The nerve terminal membranes were resuspended in a pre-incubation solution containing 50 mM Tris, 1 mM EDTA, 2 mM EGTA, pH 7.4, with adenosine deaminase (2 U/ml) and incubated at 37 °C for 30 min to remove endogenous adenosine. The suspension was centrifuged for 20 min at 14,000×g and the pellet resuspended in Tris/Mg solution with 4 U/ml of adenosine deaminase to evaluate A_1R binding parameters, as previously described (Rebola et al., 2003a). Briefly, for saturation binding curves, synaptosomal membranes (200 μl, ~60 μg protein) were incubated in a 96 well-plate, with five concentrations (0.1–10 nM) of [^{3}H]DPCPX, a selective A_1R antagonist, in Tris/Mg solution in a final volume of 300 μl. For displacement binding curves, synaptosomal membranes (200 μl, ~60 μg protein) were incubated in a 96 well-plate with 2 nM [^{3}H]DPCPX and 10 different concentrations of the selective A_1R agonist, CPA (ranging from 0.1 nM to 0.1 μM), in Tris/Mg solution, in a final volume of 300 μl. After 2 h of incubation at room temperature (22–25 °C) binding reactions were stopped by vacuum filtration through glass fiber filters (filtermats from Skatron) using a Skatron 1719 cell harvester and washed with 5 ml of Tris/Mg solution at 4 °C. After drying, the filters were placed in scintillation vials with 5 ml of scintillation liquid (Optiphase HiSafe scintillation Cocktail from Pharmacia). Radioactivity was determined after 12 h with an efficiency of 55–65% for 2 min. Results are expressed as specific binding, determined by subtraction of the non-specific binding, which was measured in the presence of 2 μM XAC, and normalized per amount of protein. All binding assays were performed in duplicate. To derive the binding parameters from saturation curves (K_D and B_{max} values) the data