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DELETION OF PRESYNAPTIC ADENOSINE A_1 RECEPTORS IMPAIRS THE RECOVERY OF SYNAPTIC TRANSMISSION AFTER HYPOXIA

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Abstract—Adenosine protects neurons during hypoxia by inhibiting excitatory synaptic transmission and preventing NMDA receptor activation. Using an adeno-associated viral (AAV) vector containing Cre recombinase, we have focally deleted adenosine A_1 receptors in specific hippocampal regions of adult mice. Recently, we found that deletion of A_1 receptors in the CA1 area blocks the postsynaptic responses to adenosine in CA1 pyramidal neurons, and deletion of A_1 receptors in CA3 neurons abolishes the presynaptic effects of adenosine on the Schaffer collateral input [J Neurosci 23 (2003) 5762]. In the current study, we used this technique to delete A_1 receptors focally from CA3 neurons to investigate whether presynaptic A_1 receptors protect synaptic transmission from hypoxia. We studied the effects of prolonged (1 h) hypoxia on the evoked field excitatory postsynaptic potentials (fEPSPs) in the CA1 region using *in vitro* slices. Focal deletion of the presynaptic A_1 receptors on the Schaffer collateral input slowed the depression of the fEPSPs in response to hypoxia and impaired the recovery of the fEPSPs after hypoxia. Delayed responses to hypoxia linearly correlated with impaired recovery. These findings provide direct evidence that the neuroprotective role of adenosine during hypoxia depends on the rapid inhibition of synaptic transmission by the activation of presynaptic A_1 receptors. **© 2005 Published by Elsevier Ltd on behalf of IBRO.**

Key words: CA1, Schaffer collaterals, electrophysiology, AAV, Cre recombinase, inducible knock-out mice.

Extracellular adenosine levels in the CNS increase after head injury, seizures, hypoxia, hypoglycemia and ischemia (Winn et al., 1979, 1981; Zhu and Krnjevic, 1993; Headrick et al., 1994; Dunwiddie, 1999). Neuronal damage in these conditions is primarily caused by excessive release of glutamate (Simon et al., 1984; Obrenovitch and Urenjak, 1997; Sattler et al., 2000), and adenosine protects neurons by activating A_1 receptors that reduce glutamate release and NMDA receptor activation (Fredholm, 1997; Sweeney, 1997; de Mendonca et al., 2000). Accordingly, protection against hypoxia and ischemia can be achieved by increasing extracellular levels of adenosine through inhibition of adenosine degradation or reuptake (Gidday et al., 1995; Miller et al., 1996; Jiang et al., 1997). Conversely, adenosine receptor antagonists or increased breakdown of extracellular adenosine exacerbates neuronal loss from hypoxia and ischemia (Sweeney, 1997; de Mendonca et al., 2000).

In the CA1 region, hypoxia and ischemia disrupt synaptic transmission, protein synthesis, maintenance of ATP levels, cytoskeletal integrity, and neuronal morphology (Lipton, 1999; Wang et al., 1999). However, hypoxia also induces release of adenosine (Dale et al., 2000; Frenguelli et al., 2003) which rapidly depresses synaptic transmission and neuronal firing (Lipton and Whittingham, 1979; Fowler, 1989; Gribkoff et al., 1990). This inhibition of neuronal activity prevents glutamatergic excitotoxicity, allowing full recovery from hypoxia (Sebastiao et al., 2001). These effects are primarily mediated by adenosine A_1 receptors because A_1 receptor antagonists or constitutive lack of A_1 receptors prevents the full recovery of synaptic activity after hypoxia (Fowler, 1989; Gribkoff et al., 1990; Johansson et al., 2001). Adenosine may protect neurons from hypoxia by preventing NMDA receptor activation (Sebastiao et al., 2001), but it remains unknown whether this protection is mediated by activation of presynaptic A_1 receptors that inhibit glutamate release.

Pharmacological tools cannot distinguish between pre- and postsynaptic A_1 receptors, but we recently developed a method to focally delete A_1 receptors in CA1 and CA3 neurons. Injection of an adeno-associated viral (AAV) vector containing Cre recombinase (AAV-Cre) into the brains of mice with loxP sites flanking the major coding exon for the A_1 receptor disrupts the A_1 gene in adult mice (Scammell et al., 2003). This focal deletion of A_1 receptors from the CA1 neurons blocks their postsynaptic responses to adenosine. In contrast, deletion of A_1 receptors from the CA3 neurons abolishes the presynaptic effects of adenosine on the Schaffer collateral input (Scammell et al., 2003). In the current study, we used the AAV-Cre technique to investigate whether presynaptic A_1 receptors protect synaptic transmission from hypoxia.

EXPERIMENTAL PROCEDURES

Animals and microinjections

Inducible A_1 receptor knock-out mice were produced using homologous recombination to introduce loxP sites around the major coding exon of the adenosine A_1 receptor gene (Scammell et al., 2003). Cre recombinase can then be introduced to delete the

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Abbreviations: AAV, adeno-associated virus; ACSF, artificial cerebrospinal fluid; CPT, 8-cyclopentyltheophylline; DMSO, dimethyl sulfoxide; fEPSPs, field excitatory postsynaptic potentials; GFP, green fluorescent protein.

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sequence between the loxP sites, producing a truncated and non-functional A_1 receptor gene.

Mice were housed in a pathogen-free barrier facility maintained at 21.5–22.5 °C with lights on at 7:00 A.M. and off at 7:00 P.M. Mice had food and water available *ad libitum*. The Institutional Animal Care and Use Committee and The Committee on Microbiologic Safety of Harvard Medical School approved all procedures in accordance with international guidelines. All efforts were made to minimize the number of animals used and their suffering. Ten adult, male mice weighing 25–35 g were anesthetized with a bolus of chloral hydrate (450 mg/kg i.p.), and 1 μl AAV-Cre was stereotaxically microinjected into the right dorsal hippocampus (1.9 mm behind bregma, 2.1 mm lateral, and 2.1 mm below the dural surface). As a control, 1 μl of an AAV expressing green fluorescent protein (AAV-GFP) was injected into the left dorsal hippocampus. To minimize tissue injury, each AAV was injected slowly over 1 h, using a pressure-injection system (Picospritzer II; General Valve, Fairfield, NJ, USA) and glass pipettes with a 10- to 20-μm diameter tip (Scammell et al., 1998).

Slice preparation and extracellular field recordings

Two to 4 weeks after the AAV injections, *in vitro* slices were prepared from inducible A_1 receptor knock-out mice. Under isoflurane anesthesia, mice were decapitated and the brain rapidly removed and placed in cold artificial cerebrospinal fluid (ACSF) containing (in mM): 128 NaCl, 3 KCl, 0.5 NaH_2PO_4, 1 $MgCl_2$, 1.5 $CaCl_2$, 23.5 $NaHCO_3$, and 10 glucose, equilibrated with 95% O_2 and 5% CO_2 (pH 7.35, 315–320 mOsm). Coronal forebrain slices containing the hippocampus (400 μm thick) were cut using a vibrating microtome (VT1000; Leica, Bannockburn, IL, USA) while maintained in ice cold oxygenated ACSF. Slices were hemisected and kept at 22 °C in oxygenated ACSF for 1 h before the recording started.

Field excitatory postsynaptic potentials (fEPSPs) were recorded from the CA1 stratum radiatum (Fig. 1A) with glass electrodes filled with 2 M NaCl (2–4 MΩ) using a Multiclamp 700A amplifier (Axon Instruments, Foster City, CA, USA). Signals were filtered at 1–2 kHz and digitized at 40 kHz with Digidata 1200 hardware and pClamp 8.2 software (Axon Instruments). Using a concentric stimulating electrode (FHC, Bowdoinham, ME, USA) placed in the stratum radiatum adjacent to the CA2 field (Fig. 1A), the Schaffer collateral fibers were stimulated every 20 s with single, constant current pulses of 0.2 ms duration. Stimulation pulses were delivered with a constant-current source (Iso-flex; A.M.P.I. Jerusalem, Israel), triggered by Clampex software (Axon Instruments), and stimulus strength was adjusted to give approximately 75% of maximum fEPSP amplitude ranging between 0.5 and 2 mV. Slices were recorded submerged and perfused (6 ml/min) with ACSF (Pearson et al., 2001) maintained at 32 °C using a temperature controller (TC-344B; Warner Instruments, Hamden, CT, USA). In every experiment, adenosine effects were measured initially, and then after the complete washout of adenosine, the response to 1 h hypoxia was tested. Hypoxia was induced using ACSF pre-equilibrated with 95% N_2 and 5% CO_2, and each slice received only one exposure to hypoxia. Stock solutions of the adenosine A_1 receptor antagonist 8-cyclopentyltheophylline (CPT) were prepared in dimethyl sulfoxide (DMSO); the final concentration of DMSO in the ACSF was $<$0.025%.

In situ hybridization

In situ hybridization for A_1 receptor mRNA was performed as previously described (Scammell et al., 2003). After electrophysiologic recordings, slices were fixed overnight in 4% paraformaldehyde and equilibrated in 20% sucrose with DEPC. To ensure that the sections were cut parallel to the slice we first cut a flat surface in the frozen embedding medium. We then laid the slice on a microscope slide and flipped the slide to attach the slice to the flat embedding medium. Sections were then cut at 30 μm, mounted on slides, and hybridized with an antisense riboprobe directed against bases 436–900 of the rat A_1 receptor cDNA sequence (Reppert et al., 1991). Deletion of A_1 receptors from the CA3 region was confirmed using film autoradiography and emulsions.

Data analysis and statistics

Evoked field potentials were quantified as the slope of the fEPSP measured between 10 and 90% of the fEPSP peak amplitude. Only data from slices with histologically confirmed deletion of A_1 receptors limited to the CA3 area and with less than 20% fEPSP inhibition by adenosine were used for the analysis of the responses to hypoxia. Only one slice from each animal met these two criteria and in every case this was the slice showing the most extended CA3 deletion. Onset of the hypoxia-mediated depression of the fEPSPs was estimated by fitting the normalized data during the 1 h hypoxia with single exponential functions with a nonlinear least-squares fitting method using IGOR Pro 4.0 (WaveMetrics, Lake Oswego, OR, USA). Since the fEPSP depression in response to hypoxia depends on both adenosine-dependent and adenosine-independent mechanisms, to calculate the time constant of the adenosine-mediated effect we assumed that adenosine-dependent and -independent effects are compounded (fEPSPslope $= e^{-t/\tau_1} \cdot e^{-t/\tau_2}$). Therefore the effective inverse decay time constant, measured in control condition can be expressed as $1/\tau_e = 1/\tau_1 + 1/\tau_2$. Data are presented as means±S.E.M. and statistical significance was established by unpaired two-tailed *t*-tests.

RESULTS

Hypoxia in the presence of A_1 receptor antagonists

Hypoxia completely depressed the hippocampal fEPSPs within 5 min in slices from wild-type mice. After 1 h of hypoxia, reoxygenation produced a complete recovery of the fEPSPs (Fig. 1). In the presence of the A_1 receptor antagonist CPT (1 μM), the fEPSPs was depressed more slowly by hypoxia than in control conditions (Fig. 1). Specifically, the time constant for the depression of the fEPSPs was 10.3±1.5 min (n=6) in the presence of CPT compared with 1.1±0.1 min (n=4) in control ACSF (P=0.0015, unpaired *t*-test). CPT also impaired the recovery of synaptic transmission (Fig. 1): 30 min after the return to normoxia, the fEPSP slopes recovered to 107±17% of baseline in control ACSF but only to 45.3±9.2% in the presence of CPT (P=0.026, unpaired *t*-test).

Hypoxia in slices with deletion of presynaptic A_1 receptors

To determine whether these neuroprotective effects of adenosine were presynaptic, we focally deleted A_1 receptors in the CA3 neurons. Ten inducible A_1 receptor knock-out mice were injected with AAV-Cre into the CA3 region to delete A_1 receptors and injected contralaterally with AAV-GFP as a control. *In situ* hybridization confirmed that focal deletion of A_1 receptors was restricted to the CA3 area in five animals.

In slices lacking A_1 receptor mRNA only in CA3, the response to adenosine was markedly reduced, and the fEPSPs were more vulnerable to hypoxia (Fig. 2). Application of adenosine (50 μM) inhibited the Schaffer collateral fEPSPs by only 14.4±3.0% (n=4) on the AAV-Cre injection side, compared with an inhibition of 57.7±4.5%

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