

Adenosine A₃ Receptors in the Rat Hippocampus: Lack of Interaction with A₁ Receptors

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ABSTRACT Adenosine acts as a neuromodulator in the hippocampus essentially through activation of inhibitory A₁ receptors. Using single-cell PCR analysis, we found that CA1 pyramidal cells coexpress A₁ receptor mRNA together with that of another adenosine receptor, the A₃ receptor. As occurs for the A₁ receptor, Western blot analysis indicated that the A₃ receptor is also located in hippocampal nerve terminals. However, activation of A₃ receptors with its purportedly selective agonist CI-IBMECA (0.1–10 μM) failed to affect hippocampal synaptic transmission or to modify the evoked release of glutamate or GABA. Also, blockade of A₃ receptors with MRS 1191 (5 μM) failed to affect either hypoxia- or ischemia-induced depression of hippocampal synaptic transmission. Activation of A₃ receptors also failed to control A₁ receptor function, as CI-IBMECA (100 nM) did not modify the ability of CPA to displace [³H]DPCPX binding to hippocampal membranes or the A₁ receptor-mediated inhibition of hippocampal synaptic transmission. However, ligand binding studies revealed that CI-IBMECA displaced the binding of an A₁ receptor agonist ([³H]R-PIA, K_i = 47 nM) or antagonist ([³H]DPCPX, K_i = 130 nM), which suggests that A₃ receptor ligands also act on native A₁ receptors. We believe that A₃ receptors are expressed in hippocampal neurons and are located in hippocampal nerve terminals, though their function remains elusive. *Drug Dev. Res.* 58:428–438, 2003. © 2003 Wiley-Liss, Inc.

Key words: cross-talk; PCR; knockout; Western blot; CI-IBMECA; MRS 1191

Adenosine is believed to act as a neuroprotective agent by activation of each of the four cloned and pharmacologically characterized adenosine receptors (A₁, A_{2A}, A_{2B}, and A₃) [Fredholm, 1996]. For example, the most recently cloned A₃ receptor has been reported to have a profound effect on the outcome of ischemic insults [reviewed by von Lubitz et al., 2001] and to protect against chemotoxicity [Fishman et al., 2001] and apoptotic cell death, namely in astrocytes [Abbraccio et al., 1998] and neurons [Sei et al., 1997]. It

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has been argued that A₃ receptors have an affinity for adenosine considerably lower ($K_i=1000$ nM) than A₁ ($K_i=10$ nM) and A_{2A} ($K_i=30$ nM) receptors [Gallo-Rodriguez et al., 1994]. This implies that the activation of A₃ receptors is essentially predicted to occur in stressful situations that lead to a marked increase in the extracellular concentration of adenosine [Jacobson, 1998]. However, more recent data indicate that adenosine is as potent on A₃ as on A₁ and A_{2A} receptors [Fredholm et al., 2001; Schulte and Fredholm, 2001].

Whether A₃ receptors might also control neuronal activity under nonstressful situations is not clear because the effects of A₃ receptor activation on neuronal activity at the cellular level have been poorly investigated. In the cerebral cortex, the A₃ receptor agonist IB-MECA inhibited synaptic transmission in a manner sensitive to an A₃ receptor antagonist [Brand et al., 2001]. In the hippocampus, A₃ receptor activation facilitates synaptic plasticity phenomena [Costenla et al., 2001] and increases neuronal activity through a protein kinase A-dependent facilitation of postsynaptic calcium currents [Fleming and Mogul, 1997], although A₃ receptors appear not to affect calcium-activated potassium currents [Dunwiddie et al., 1999]. In the hippocampus, A₃ receptor immuno-reactivity was identified in nerve terminals [Díaz-Hernández et al., 2002] and the activation of these putative presynaptic A₃ receptors inhibits both metabotropic glutamate group III receptor- [Macek et al., 1998] and adenosine A₁ receptor-mediated responses [Dunwiddie et al., 1997].

In spite of these evidences for both presynaptic and postsynaptic A₃ receptor-mediated effects in the central nervous system, the density of A₃ receptors in the brain is rather low and the identification of A₃ receptors has mostly been accessed by whole-tissue PCR, which has revealed the presence of mRNA encoding for A₃ receptor in the hippocampus, cerebellum, and striatum [De et al., 1993; Dixon et al., 1996; Richardson et al., 2000]. However, *in situ* hybridization studies have questioned whether neurons in the brain expressed A₃ receptors [Rivkees et al., 2000].

Thus, we now took advantage of single-cell PCR and combined biochemical and immunological techniques to test if hippocampal neurons are endowed with A₃ receptors. We tried to define the possible role of A₃ receptors in the hippocampus in nonstressful and in stressful situations, with particular attention to whether A₃ receptors control the predominant adenosine A₁ receptors.

MATERIALS AND METHODS

Single-Cell PCR

Sections 10 μ m thick from male Wistar rats frozen brains were obtained with a cryostat, taken between

–4.52 and –2.80 mm from Bregma, dehydrated in 100% ethanol, rehydrated gradually (50–100% ethanol), stained with Nissl stain (gives purple color to neurons), and dehydrated again with xylene according to Luo et al. [1990]. The required neurons from CA1 area of the hippocampus were laser microdissected with an Arcturus LCM 210 PixCell II system with a spot size of 7.5 μ m. The total RNA was then extracted according to Absolutely RNA Nanoprep Kit instructions from Stratagene and subjected to reverse transcription (RT) and cDNA amplification. For RT and cDNA amplification, we used the 3'-end amplification (TPEA) protocol previously described by Dixon et al. [1998]. All the primers used were from Perkin Elmer Applied Biosystems (United Kingdom). The amplified samples (5 μ l aliquots) were used for subsequent gene-specific PCR after cDNA purification according to QIAquick spin handbook (QIAGEN). For gene-specific PCR, the samples of the preamplified cDNA were subjected to 45 rounds of PCR in 20 μ L of 45 mM Tris-HCl (pH 8.1), 12.5% (w/v) sucrose, 12 mM (NH₄)₂SO₄, 3.5 mM MgCl₂, and 0.5 mM deoxynucleotide triphosphates, with 100 ng of the forward and reverse primer. The cycling conditions were 2.5 min at 92°C (denaturation), 1.5 min at 60°C (annealing), and 1 min at 72°C (extension). After amplification, the products were separated on 2% agarose gels and visualized using ethidium bromide. The primers used are listed in Table 1. In all experiments, positive controls for the primers used were electrophoresed in parallel to the gene-specific assays. These routinely contained cDNA derived from 1 ng of whole-brain total RNA.

Western Blot Analysis in Nerve Terminal Membranes

The Western blot analysis of A₃ receptors was carried out in membranes from a Percoll-purified synaptosomal fraction, prepared as previously described [Cunha et al., 1996]. After determining the amount of protein [Spector, 1978], each sample was diluted with an equal volume of 2 \times SDS-PAGE sample buffer (62.5 mM Tris-HCl pH 6.8, 25% (v/v) glycerol, 2% (w/v) SDS, 0.01% (w/v) bromophenol blue, 5% (v/v) β -mercaptoethanol). These diluted samples and the prestained molecular weight markers (Amersham) were separated by SDS-PAGE (10% resolving gel and 4% concentrative gel) under reducing conditions and electrotransferred to polyvinylidene difluoride membranes (0.45 μ m from Amersham). After blocking for 2 h at room temperature with 5% milk in Tris-buffered saline (140 mM NaCl, 20 mM Tris), pH 7.6 containing 0.1% Tween 20 (TBS-T), the membranes were incubated overnight at 4°C with a rabbit anti-adenosine A₃ receptor affinity purified

TABLE 1. PCR Primers Used

Name	Abbreviation	Gene name	Primers	PCR product length (bp)
Glial fibrillary acidic protein	GFAP		68–87, 216–198	170
Intronic marker	ET3	Region chromosome 3		149
Adenosine receptors:				
A ₁	A ₁	<i>Adora1</i>	1225–1242, 1392–1373	168
A ₃	A ₃	<i>Adora3</i>	1590–1608, 1739–1719	150
NMDA receptor subunit 1	NR1	<i>Grin1</i>	3941–3932, 4091–4074	178

polyclonal antibody (Chemicon, 1 mg/mL, 1:1000 dilution). After four 10-min washes with TBS-T containing 0.5% milk, the membranes were incubated with the alkaline phosphatase-conjugated anti-rabbit secondary antibody (1:5000 dilution from Amersham) in TBS-T containing 1% milk during 90 min at room temperature. After five 10-min washes in TBS-T containing 0.5% milk, the membranes were incubated with Enhanced Chemi-Fluorescence (ECF) for 5 min and then analyzed densitometrically with a Storm (Molecular Devices).

Electrophysiological Recordings in Hippocampal Slices

Male Wistar rats (5–6 weeks old), handled according to the European guidelines (86/609/EEC), were decapitated after halothane anesthesia, and the hippocampus dissected free in ice-cold Krebs solution of the following composition (mM): NaCl 124, KCl 3, NaHCO₃ 26, Na₂HPO₄ 1.25, MgSO₄ 1, CaCl₂ 2, glucose 10, gassed with a 95% O₂ + 5% CO₂ mixture. Slices were cut (400 μm) with a McIlwain tissue chopper and allowed to recover for at least 1 h in a resting chamber within the same gassed medium at room temperature (20–25°C). Individual slices were transferred to a submersion recording chamber (1 mL capacity) and continuously superfused at a rate of 3 mL/min with the same gassed Krebs solution at 30.5°C or at 32°C for hypoxia and ischemia experiments. Stimulation was delivered to the Schaffer collateral/commissural fibers through a bipolar concentric wire electrode and rectangular pulses of 0.1 ms duration were applied every 15 sec. The initial intensity of the stimulus was chosen to elicit a response of about 50% of maximal field excitatory postsynaptic potential (fEPSP) amplitude. The fEPSPs were recorded extracellularly from CA1 stratum radiatum by use of micropipettes filled with NaCl 4 M with 2–4 MΩ resistance, and displayed on a Tektronix digitizing oscilloscope. The averages of eight consecutive responses were obtained, graphically plotted, and recorded for further analysis with the LTP software [Anderson and Collingridge, 1997]. The slopes and amplitudes of the averaged fEPSPs were determined

and then averaged during the predrug control, drug superfusion, and postdrug washout period; at least six responses were included in each average. Compounds were added through the superfusion solution.

The hypoxic insult consisted of changing the superfusion tube to a Krebs solution in which the bubbling gas was 95% N₂+5% CO₂ for 4 min and the maximum depression in amplitude of the fEPSPs registered. The ischemic insult was achieved by changing for 10 min the superfusion tube to a Krebs solution, but where glucose was not present, gassed with 95% N₂+5% CO₂. After the challenging periods, the superfusion solutions were changed back to normal Krebs solution. The data were analyzed as mean percentage change in response amplitude when compared with responses obtained during the control period. The values are shown as mean ± SEM of *n* (number of experiments), except where otherwise indicated. The significance of differences was evaluated by the paired Student's *t* test. *P* values less than 0.05 were considered significant.

Neurotransmitter Release from Hippocampal Nerve Terminals

The evoked release of [³H]glutamate was carried out as previously described [Lopes et al., 2002], and the release of [³H]GABA was performed as described by Cunha and Ribeiro [2000]. Synaptosomes were purified by sucrose/Percoll gradients, loaded with [³H]glutamate or [³H]GABA, and placed over GF/B filters under superfusion. The synaptosomes were stimulated twice with 20 mM K⁺ for 30 sec (for glutamate release) or for 2 min (for GABA release) with a time span of 9–16 min. Drugs were added 2 min before the second stimulation period, and their effect was evaluated by changes in the evoked release of tritium measured in the effluent.

Membrane Binding Experiments

Either membranes or synaptosomes were prepared as previously described [Lopes et al., 1999]. Competition curves of the adenosine A₁ receptor antagonist [³H]DPCPX by the adenosine A₁ receptor agonist CPA were performed in the absence and in the

presence of the adenosine A₃ receptor agonist Cl-IBMECA. Competition curves of the A₁ receptor antagonist [³H]DPCPX, and of the A₁ receptor agonist [³H]PIA by the A₃ agonist Cl-IBMECA, were also performed. The appropriate synaptosome or membrane preparation (120 μL containing 165–331 μg protein) was incubated with either [³H]DPCPX (2 nM) or [³H]R-PIA (2 nM) and 10 different concentrations of the displacer Cl-IBMECA (ranging from 0.1 nM to 10 μM) or CPA (ranging from 0.1 nM to 1 μM) in 50 mM Tris and 2 mM MgCl₂, pH 7.4 (if membranes) or Krebs solution (if synaptosomes) in a final volume of 300 μL. All samples were assayed in triplicate. Nonspecific binding was evaluated in the presence of 2-chloroadenosine (CADO, 100 μM) and represented 10–15% of total binding. The test tubes were incubated for 2 h at room temperature (20–25°C) for [³H]DPCPX binding or 2 h at 37°C for [³H]R-PIA binding. The binding reactions were stopped by vacuum filtration through glass fiber filters (filtermats to receptor binding from Skatron) using a Skatron 1719 cell harvester, and the tubes and filters washed with 5 mL of the respective ice-cold incubation buffer. The filters were then placed in scintillation vials and 4 mL of scintillation liquid (Optiphase HiSafe Scintillation Cocktail) added. Radioactivity was determined after at least 12 h with a counting efficiency of 55–60%. The protein concentration was determined using the Bio-Rad protein assay [Bradford, 1976]. The IC₅₀ values were converted into K_i values upon nonlinear fitting of the semilogarithmic curves derived from the competition curves. An *F* test (*P* < 0.05) was used to determine whether the competition curves were best fitted by a one or two independent binding site equation. The K_i values are presented as mean with 95% confidence interval (CI).

Autoradiographic Experiments

Six mice of each genotype, wild-type (A₃R^(+/+)), heterozygous (A₃R^(+/-)), and mice lacking the A₃ receptor (A₃R^(-/-)), on a 129/B6D2 background [Salvatore et al., 2000] (generation F2 or F3), were anesthetized with carbon dioxide and thereafter decapitated. The brains were immediately dissected out, frozen on dry ice, and stored at -80°C until used. Using a cryostat, the levels of Bregma 1.42 to 0.50, -0.34 to -0.58, -1.70 to -2.18, and -7.08 to -7.48 mm [Franklin and Paxinos, 1997] were sectioned into 10-μm-thick frontal sections, which were thaw mounted on polylysine-coated glass slides (Menzel-Gläser, Germany). Slides were stored at -20°C until used.

Receptor autoradiography was performed by drying the sections at room temperature for 60 min and preincubating at 37°C in 170 mM Tris-HCl, 2 U/mL adenosine deaminase (ADA) and 1 mM EDTA, pH

7.4, for 30 min. After washing twice at room temperature in 170 mM Tris-HCl with 1 mM MgCl₂ (pH 7.4) for 10 min, the sections were incubated for 2 h at room temperature in a solution containing 170 mM Tris-HCl (pH 7.4), 1 mM MgCl₂, 2 U/mL ADA, and 0.2, 0.5, 1, 2, 5, or 10 nM [³H]DPCPX, and for nonspecific binding, 20 μM R-PIA. The slides were then washed twice in 170 mM Tris-HCl (pH 7.4) at 4°C for 5 min and dipped three times in ice-cold distilled water. Slides were dried overnight in front of a fan at 4°C. Sections were then apposed to Hyperfilm-³H (Amersham) together with tritium standards (Amersham) for 3 weeks. The optical density was measured using a computer program (MCID, Imaging Research, St. Catherines, Canada), and values were converted to fmol/mg grey matter using the tritium standards. The specific binding was achieved by subtracting the nonspecific binding from the total binding values.

Compounds

3-Ethyl-5-benzyl-2-methyl-4-phenylethynyl-6-phenyl-1,4-(±)-dihydropyridine-3,5-dicarboxylate (MRS 1191), N⁶-cyclopentyladenosine (CPA), and 1,3-dipropyl-8-cyclopentylxanthine (DPCPX) were purchased from Sigma-RBI (Natick, USA). 1-[2-Chloro-6-[[3-iodophenyl)methyl]amino]-9H-purin-p-yl]-1-deoxy-N-methyl-b-d-ribofuranuronamide (2-Cl-IBMECA) and 4-(2-[7-amino-2-[2-furyl]{1,2,4}triazolo[2,3-a]{1,3,5}triazin-5-yl-amino]ethyl)phenol (ZM 241385) were from Tocris (Bristol, UK). 2-Chloroadenosine was from the Sigma (Poole, Dorset, UK). [³H]DPCPX (specific activity: 110.6 Ci/mmol) was from DuPont-NEN (Stevenage, UK) and adenosine deaminase (from calf intestine, 200 U/mg protein, 2 mg/10 mL solution in 50% glycerol (v/v), 10 mM potassium phosphate, pH 6.0) was from Boehringer (Manheim, Germany). [³H]glutamate (specific activity: 45 Ci/mmol), [³H]GABA (specific activity: 76.2 Ci/mmol), and [³H]R-phenylisopropyladenosine ([³H]R-PIA; specific activity: 63 Ci/mmol) were from Amersham (Buckinghamshire, UK). All drugs were diluted daily into the appropriate media from 5 mM stock solutions made up in dimethylsulfoxide stored at -20°C, except adenosine deaminase, [³H]R-PIA and [³H]DPCPX, which were prepared directly into the incubation solution each day. DPCPX was made up as a 5 mM stock solution in dimethylsulfoxide 99% (v/v) containing 0.01 M NaOH.

RESULTS

Presence of Adenosine A₃ Receptors in Hippocampal Neurons Together with A₁ Receptors

Taking advantage of laser dissection, which allows isolated cells to be taken from heterogeneous tissue

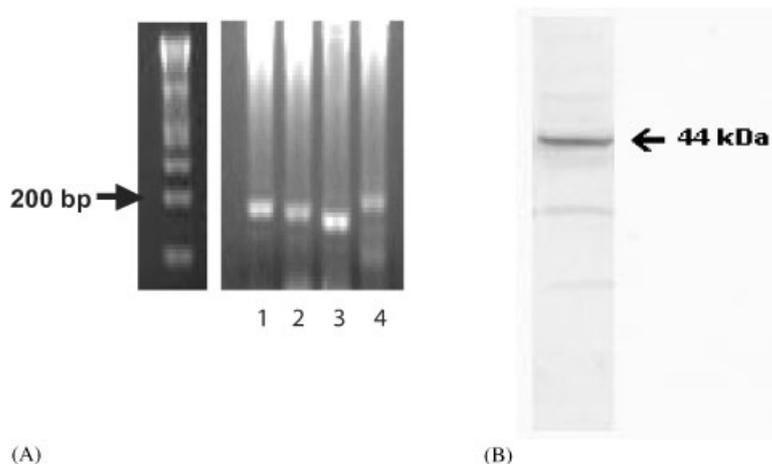


Fig. 1. Presence of adenosine A_3 receptors in the hippocampus. (A) mRNA profile for adenosine receptors in a single pyramidal neuron from CA1 area of the hippocampus. The first lane from the left corresponds to ladder standards: 1, A_1 receptor; 2, A_{2A} receptor; 3, A_3

receptor; and 4, NMDA receptor subunit 1. (B) Western blot analysis of A_3 receptor immuno-reactivity in membranes from purified nerve terminals of the rat hippocampus (100 μ g of membrane protein applied to the gel).

with little contamination from the surroundings, we dissected single pyramidal neurons from the CA1 area of the hippocampus and subjected them to gene-specific PCR. In addition to the adenosine receptors mRNAs, we also tested NMDAR1, a marker of pyramidal cells, GFAP, an astrocyte marker, and one intronic marker to ensure the quality of the RT reaction. From 10 cells analyzed that were NMDAR1-positive, five of them showed coexpression of mRNA encoding for adenosine A_1 and A_3 receptors (see Fig. 1A). We are aware that contamination from, for example, microglial processes cannot be excluded.

Given this apparent coexpression of A_1 and A_3 receptor mRNA in pyramidal cells, we looked for the presence of A_3 receptors where A_1 receptors are most abundant (i.e., in nerve terminals). As illustrated in Figure 1B, there was a clear A_3 receptor-like immunoreactivity that displayed an electrophoretic mobility corresponding to an apparent molecular weight of 44 kDa, similar to the expected molecular weight of the rat A_3 receptor [Christofi et al., 2001]. This signal was not observed either when the primary antibody was not added or when this experiment was carried out in the presence of 1 μ g of the immunizing peptide.

Effects of A_3 Receptors on Synaptic Transmission and Neurotransmitter Release

The purportedly more selective adenosine A_3 receptor agonist available, CI-IBMECA, did not affect synaptic transmission in the concentration range tested (10 nM to 10 μ M) (Fig. 2). This lack of effect of A_3 receptor activation could be caused by opposite effects on glutamate and GABA release. We therefore evaluated the effect of A_3 receptor activation on

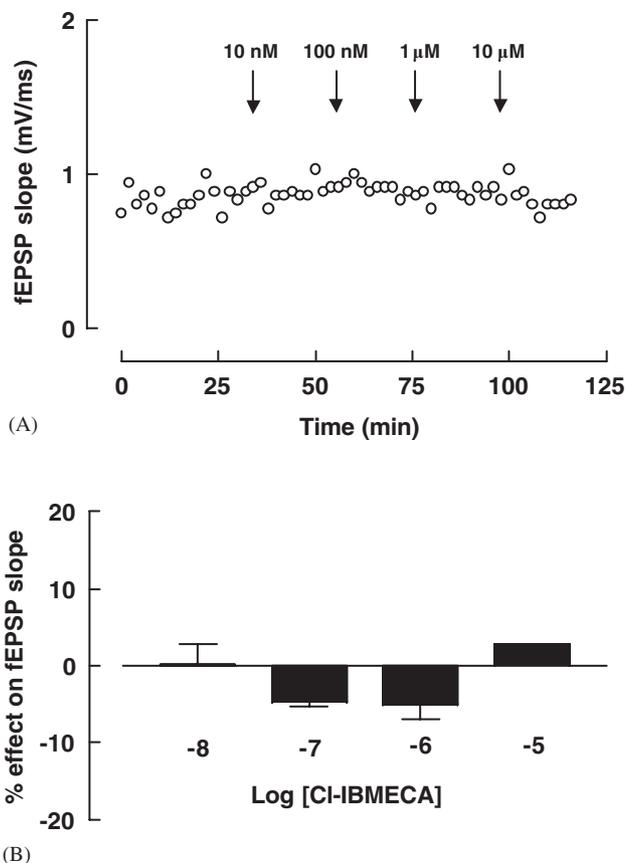


Fig. 2. Effect of an A_3 receptor agonist CI-IBMECA on synaptic transmission in rat hippocampal slices. (A) Time course of the averaged slope of eight consecutive fEPSPs in a representative experiment where a hippocampal slice was successively superfused with increasing concentrations of CI-IBMECA at the time indicated by the arrows. (B) Summary data showing the percentage effect of CI-IBMECA on synaptic transmission. Results are means \pm SE of two experiments.

TABLE 2. Effect of A₃ Receptor Activation on Glutamate and GABA Release from Hippocampal Nerve Terminals

	Control	Cl-IBMECA (100 nM)	Control	Cl-IBMECA (1–10 μM)
[³ H] glutamate release	0.81 ± 0.01 (n=4)	0.74 ± 0.12 (n=4)	1.1 ± 0.3 (n=4)	0.9 ± 0.2 (n=4)
[³ H] GABA release	0.78 ± 0.04 (n=6)	0.72 ± 0.05 (n=6)	0.73 ± 0.04 (n=6)	0.77 ± 0.07 (n=6)

These experiments were conducted under blockade of A₁ receptors (using DPCPX, 50 or 200 nM) and A_{2A} receptors (using ZM 241385, 50 or 200 nM).

glutamate and GABA release from hippocampal nerve terminals. Because we had previously observed that Cl-IBMECA might activate adenosine A₁ receptors [Costenla et al., 2001], the following experiments were conducted under blockade of A_{2A} and A₁ receptors to eliminate possible nonselective responses, using the A₁ receptor antagonist DPCPX (50 nM) and the A_{2A} receptor antagonist ZM 241385 (50 nM). Cl-IBMECA (100 nM and 10 μM) had no effect on glutamate release compared to control conditions (Table 2). Likewise, 100 nM and 1 μM Cl-IBMECA failed to modify the evoked release of GABA from superfused hippocampal synaptosomes (see Table 2).

To test whether A₃ receptors might be tonically activated in stressful situations, we tested the consequences of blocking A₃ receptors on synaptic transmission during a hypoxic insult lasting 4 min. As illustrated in Figure 3A, the removal of oxygen causes a marked inhibition of synaptic transmission that recovers completely once oxygen is reintroduced. It is also evident from Figure 3A that the hypoxia-induced depression of fEPSPs was similar in both the absence and presence of the A₃ receptor antagonist MRS 1191 (5 μM). The consequences of blocking A₃ receptors were also tested in an ischemic situation applied for 10 min. But again, the ischemia-induced depression of fEPSPs was similar in the absence and in the presence of MRS 1191 (5 μM) (Fig. 3B). In control experiments, we confirmed that two consecutive hypoxic or ischemic insults caused a similar depression of fEPSPs in the absence of drugs (data not shown).

Effect of Adenosine A₃ Receptor Activation on A₁ Receptor Function

One possibility to reconcile the evidence for the neuronal location of A₃ receptors with the lack of effect of A₃ receptors on synaptic transmission and neurotransmitter release would be that the A₃ receptors mostly fulfilled a fine-tuning role, controlling the action of other neuromodulatory systems [Sebastião and Ribeiro, 2000]. Thus, we investigated if A₃ receptors could control A₁ receptor function, as previously proposed [see Dunwiddie et al., 1997]. To carry out this study, we first had to evaluate the real selectivity of the most selective A₃ receptor agonist, Cl-IBMECA,

against hippocampal A₁ receptors, because the initial selectivity of this ligand has mostly been determined using heterologously expressed adenosine receptors and previous studies have claimed that Cl-IBMECA could bind and activate A₁ receptors in the rat brain [Rivkees et al., 2000; Costenla et al., 2001].

We performed displacement binding curves of the adenosine A₁ receptor agonist [³H]R-PIA, and of the adenosine A₁ receptor antagonist [³H]DPCPX by the A₃ receptor agonist Cl-IBMECA. As illustrated in Figure 4, Cl-IBMECA (0.1 nM to 10 μM) displaced [³H]R-PIA (2 nM) with a K_i of 47 nM (95% confidence interval: 39–57 nM, n = 4) and displaced [³H]DPCPX (2 nM) with a K_i of 130 nM (95% confidence interval: 91–190 nM, n = 4). Thus, to evaluate the effect of A₃ receptor activation on the affinity of A₁ receptors, we tested the effect of a concentration of 100 nM of Cl-IBMECA on the ability of the A₁ receptor agonist CPA to displace the A₁ receptor antagonist [³H]DPCPX (2 nM). These experiments were carried out in hippocampal synaptosomes to preserve intracellular transducing pathways eventually required for the interaction to occur [Lopes et al., 1999]. In control conditions (i.e., with no Cl-IBMECA), the displacement binding curve by CPA (0.1 nM to 1 μM) was fitted best by a single binding site equation with a K_i of 1.4 nM (95% confidence interval: 0.9–2.2 nM, n = 6) (see Fig. 4C). Cl-IBMECA (100 nM) did not significantly (*P* > 0.05) change the displacement binding curve of CPA, resulting in a K_i of 2.3 nM (95% confidence interval: 1.7–3.2 nM, n = 6) (see Fig. 4C).

Because these experiments in synaptosomes were conducted in a time scale of minutes and the A₃/A₁ receptor interaction could take longer to be measured, we compared by autoradiography the binding of the selective A₁ receptor ligand [³H]DPCPX in sections from wild-type and A₃ receptor knock-out mice. Using mutant mice in which the gene for A₃ receptor was knocked out [Salvatore et al., 2000] or reduced by half (A₃R^(+/-)), we observed that no differences existed in the binding of DPCPX to the hippocampus, compared to wild-type mice (Fig. 5). There was also no difference in DPCPX binding in any other region studied (hippocampus, cortex, cerebellum, and striatum; data not shown). The B_{max} values in the CA1 region for

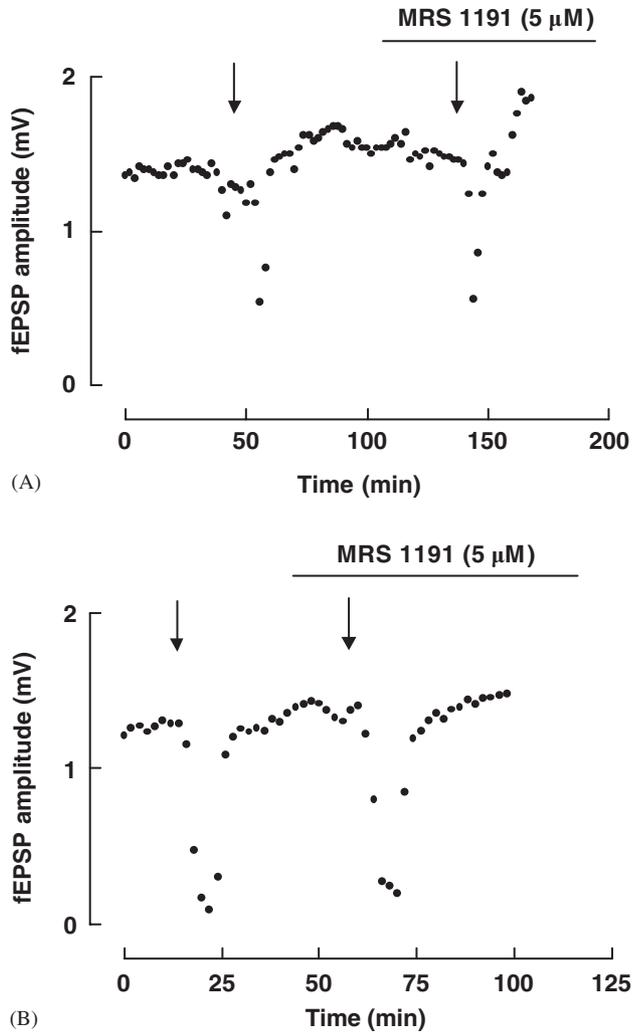


Fig. 3. Effect of an A_3 receptor antagonist on hypoxia- or ischemia-induced depression of synaptic transmission in rat hippocampal slices. (A) Time course of the averaged amplitude of eight consecutive fEPSPs in a representative experiment of hypoxia-induced depression of synaptic transmission. The hypoxia period started at the time indicated by each arrow and lasted for 4 min. The A_3 receptor antagonist MRS 1191 ($5 \mu\text{M}$) was applied during the period indicated by the horizontal line. (B) Time course of the averaged amplitude of eight consecutive fEPSPs in a representative experiment of ischemia-induced depression of synaptic transmission. The ischemia period started at the time indicated by each arrow and lasted for 10 min. The A_3 receptor antagonist MRS 1191 ($5 \mu\text{M}$) was applied during the period indicated by the horizontal line.

$A_3R^{+/+}$, $A_3R^{+/-}$, and $A_3R^{-/-}$ were 328.0 (95% confidence interval: 292.1 – 363.9), 350.7 (317.8 – 383.6), and 336.5 (306.1 – 366.9) fmol/mg grey matter, respectively, and the K_D values were 0.46 (95% confidence interval: 0.25 – 0.67), 0.53 (0.33 – 0.73), and 0.48 (0.30 – 0.66) nM, respectively ($n = 6$ for all). In the CA_3 area, the B_{max} values were 328.3 (95% confidence interval: 290.8 – 365.8), 350.0 (315.3 – 384.6), and 323.7

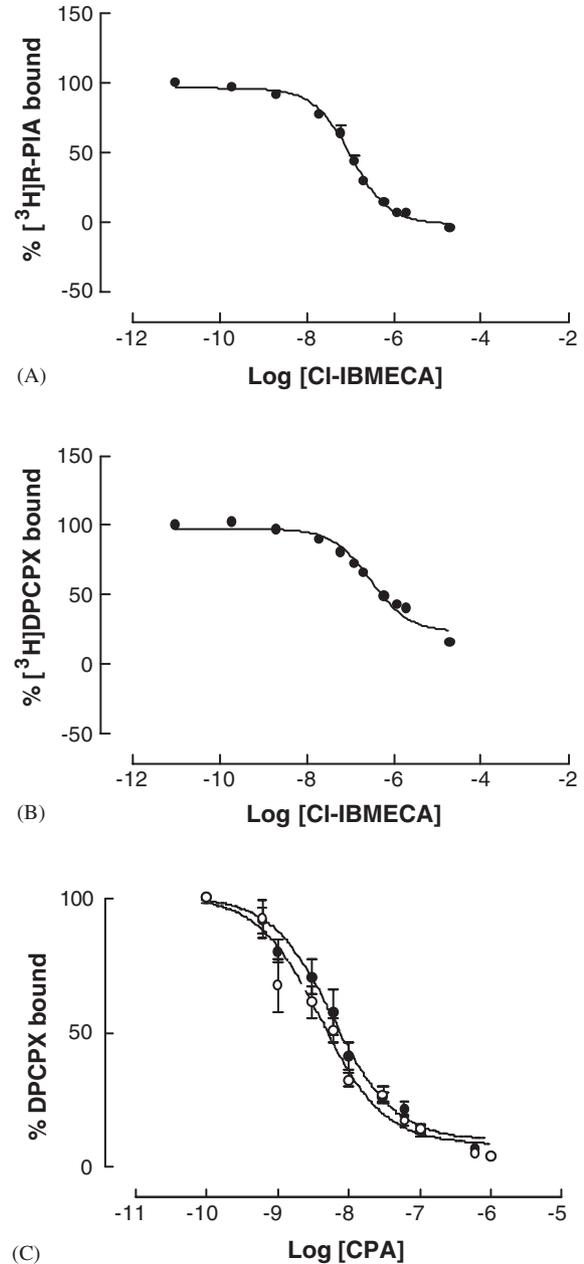


Fig. 4. Displacement binding curves of (A) [^3H]R-PIA (2 nM) and (B) [^3H]DPCPX (2 nM) by CI-IBMECA (0.1 nM to $10 \mu\text{M}$) in hippocampal membranes of young adult rats; (C) Displacement binding curves of [^3H]DPCPX (2 nM) by CPA (0.1 nM to $1 \mu\text{M}$) in the absence (\circ) and in the presence (\bullet) of the A_3 receptor agonist CI-IBMECA (100 nM). The ordinates represent the percentage of radioactive ligand bound obtained on subtraction of the nonspecific binding, determined in the presence of $100 \mu\text{M}$ CADO, from the total binding. Each point is the mean \pm SEM of four experiments performed in triplicate. The K_i values were obtained from the curves represented, which were fitted to the experimental data.

(291.0 – 356.5) fmol/mg grey matter for $A_3R^{+/+}$, $A_3R^{+/-}$, and $A_3R^{-/-}$, respectively, and the K_D values were 0.53 (95% confidence interval: 0.29 – 0.78), 0.59

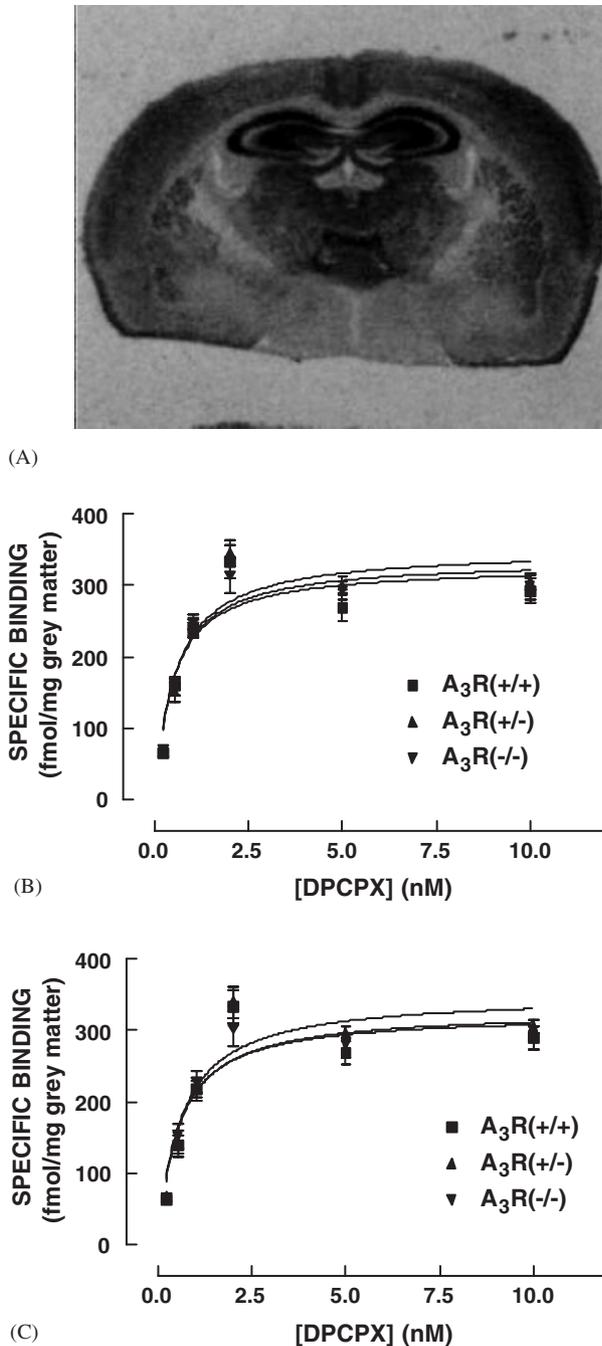


Fig. 5. The binding of [³H]DPCPX is unaffected in mice lacking A₃ receptors (A₃R^{-/-}). (A) Autoradiogram of a frontal section from a A₃R^{-/-} incubated with 2 nM [³H]DPCPX. There was no difference in [³H]DPCPX binding in the hippocampus as measured in areas CA1 (B) or CA3 (C) between the different genotypes (A₃R^{-/-}, A₃R^{+/-}, and A₃R^{+/+}). The values presented are mean ± SEM, n=6 for all.

(0.36–0.82), and 0.50 (0.30–0.71) nM, respectively (n = 6 for all).

Because the A₃/A₁ interaction was reported to occur at the functional rather than the molecular level [Dunwiddie et al., 1997], we used extracellular

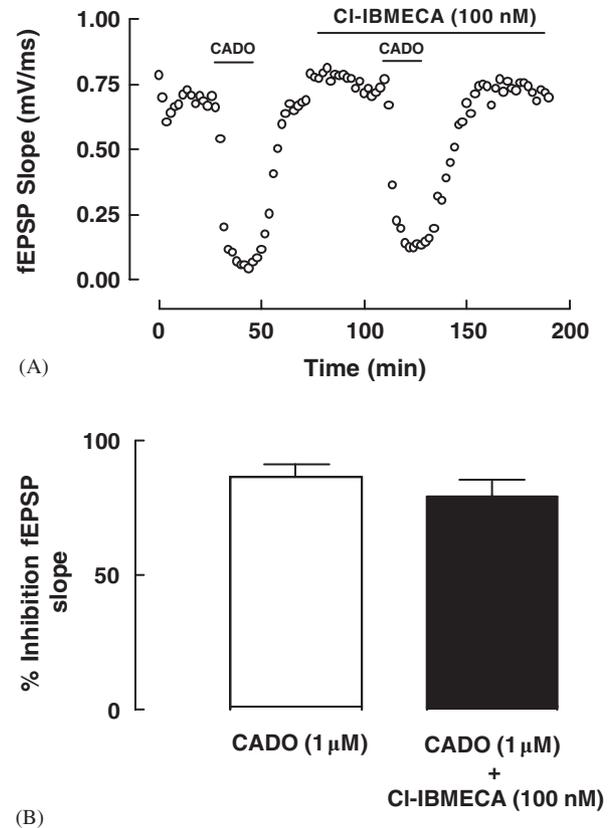


Fig. 6. Effect of A₃ receptor activation on A₁ receptor-mediated responses in the hippocampus. (A) Time course of a representative experiment. After obtaining a stable fEPSP response, CADO (1 μM) was added to the superfusion solution and then washed out. To test the effect of CI-IBMECA (100 nM), this drug was added at least 30 min before the second application of CADO. The horizontal bars represent the period of application of each drug to the superfusion solution. (B) Average percentage inhibition of fEPSP slope by CADO in the absence and in the presence of CI-IBMECA. The values are mean ± SEM of three experiments.

electrophysiological recordings from CA1 area of rat hippocampal slices to probe this interaction. We compared the effect of the stable adenosine analogue, 2-chloroadenosine (CADO) in the absence and in the presence of CI-IBMECA. CADO (1 μM) alone caused an 86.3 ± 4.5% (n = 3) inhibition of fEPSP amplitude, an effect caused by A₁ receptor activation [de Mendonça and Ribeiro, 1990]. In the presence of CI-IBMECA (100 nM), CADO (1 μM) still reduced by 79.0 ± 6.5% (n = 3) the fEPSP amplitude (Fig. 6), a value similar (*P* > 0.05) to that caused by the first application of CADO (1 μM) alone in the same slices. In control experiments, we confirmed that two successive applications of CADO (1 μM) alone elicited a similar inhibition of fEPSPs amplitude (the ratio between CADO inhibitory effect elicited by the second and first application was 1.02 ± 0.03, n = 2).

DISCUSSION

The present results provide evidence that adenosine A_3 receptors are expressed in hippocampal neurons and that they are located in hippocampal nerve terminals. However, these presynaptic A_3 receptors do not have a direct effect on synaptically evoked excitatory responses, as observed previously in the same preparation [Dunwiddie et al., 1997] and as reported to occur in the cortex [Brand et al., 2001]. Even in situations of cellular stress, such as hypoxia or ischemia, when the extracellular levels of adenosine rise [reviewed by Fredholm, 1996; Cunha, 2001], no effect of endogenous adenosine-activating A_3 receptors could be detected in the control of synaptic transmission. Also, no effects resulting from A_3 receptor activation could be monitored in the evoked release of glutamate or GABA from synaptosomes. These results are in complete agreement with the recent finding that adenosine has no effect on excitatory neurotransmission in the hippocampus of mice that lack the adenosine A_1 receptor [Johansson et al., 2001].

This lack of evident effects of presynaptic A_3 receptors also appears at odds with the previously reported ability of A_3 receptors to facilitate long-term potentiation (LTP) and long-term depression (LTD) [Costenla et al., 2001]. But taking the data together, it is likely that the control by A_3 receptors of synaptic plasticity might result from the activation of post-rather than presynaptic A_3 receptors. This would reconcile the presently observed expression of A_3 receptor mRNA in hippocampal pyramidal neurons with the previously reported ability of A_3 receptors to facilitate postsynaptic calcium currents [Fleming and Mogul, 1997]. Further work is required to characterize these postsynaptic A_3 receptors in the hippocampus.

Thus, the possible role of the identified presynaptic A_3 receptors in the rat hippocampus remains essentially elusive. It is possible that presynaptic A_3 receptors might be especially important in a particular subtype of hippocampal nerve terminals. In this respect, it has been reported that A_3 receptor activation has a minor role in the control of serotonin release in the hippocampus [Okada et al., 1997], but to the best of our knowledge no information is available on the ability of A_3 receptors to control noradrenaline, dopamine, or acetylcholine release in the hippocampus. Another possible role for these presynaptic A_3 receptors might be their ability to act as fine-tuning neuromodulators [Sebastião and Ribeiro, 2000]. In fact, A_3 receptors have been described to inhibit the actions of group III presynaptic metabotropic glutamate receptors in the hippocampus [Macek et al., 1998], to inhibit adenosine

polyphosphate-mediated presynaptic calcium transients [Diíz-Hernández et al., 2002], and also to inhibit adenosine A_1 receptor-mediated responses in the rat hippocampus [Dunwiddie et al., 1997].

We explored this possible role of A_3 receptors to attenuate A_1 receptor-mediated responses [Dunwiddie et al., 1997] and we provide the required structural background for this interaction to occur (i.e., the presence of mRNA for both receptor subtypes in the same cell), as suggested by PCR analysis of a single CA1 neuron. However, we found no apparent control by A_3 receptors of A_1 receptor-mediated inhibition of excitatory synaptic transmission or of the affinity of A_1 receptors, as shown by binding studies. Also, the mice lacking A_3 receptors displayed no differences in the number or affinity of A_1 receptors, suggesting that no long-term control by A_3 receptors of the density of A_1 receptors takes place. The concentration of the A_3 receptor agonist Cl-IBMECA used in the present study was lower than that used in previous studies because recent data show a lack of selectivity of A_3 receptor agonists over A_1 receptors [Rivkees et al., 2000; Costenla et al., 2001]. We confirmed that Cl-IBMECA binds to A_1 receptors even at low nanomolar concentrations. This obviously compromises the possibility of any eventual A_1/A_3 receptor interaction to be fully investigated and casts strong doubts on whether the previously reported A_3/A_1 receptor interaction might result from the lack of selectivity of the A_3 receptor ligands available.

One of the key questions raised by the present results is the usefulness of the A_3 receptor ligands, particularly Cl-IBMECA, to study A_3 receptor-mediated effects. The initial selectivity essays of Cl-IBMECA for A_3 receptors versus A_1 receptors were conducted in cortical tissue [Kim et al., 1994], which might behave differently [cf. Brand et al., 2001 and present data]. Also, the fact that these ligands were synthesized based upon their potency in inhibiting adenylate cyclase in heterologous expression systems [Kim et al., 1994] limits the direct comparison of the obtained parameters to native tissues, as most of the evidence seems to indicate that A_3 receptors in the brain might not couple to adenylate cyclase but rather to protein kinase C [Abbracchio et al., 1995; Dunwiddie et al., 1997; Macek et al., 1998]. Thus, the real selectivity of the currently available A_3 receptor ligands against native adenosine receptors remains to be fully evaluated, and their use in physiological experiments in native tissue must be carefully evaluated.

In conclusion, A_3 receptors are present in the hippocampus, particularly in nerve terminals, and colocalized with A_1 receptors in pyramidal neurons. The present data suggest that no interaction between

these two receptors subtypes exist and that A₃ receptors do not affect synaptic transmission (basal or in stressful conditions) or control glutamate or GABA release. That the known A₃ receptor ligands bind A₁ receptors represents a limitation for conclusive studies. The definitive answer to the function of A₃ receptors has to wait for reliable ligands and reliable functional assay to be used for the pharmacological characterization of A₃ receptor ligands vis-à-vis native A₃ receptors.

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