

# Modulation of Hippocampal Glutamatergic Transmission by ATP Is Dependent on Adenosine A<sub>1</sub> Receptors

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## ABSTRACT

Excitatory glutamatergic synapses in the hippocampal CA1 region of rats are potently inhibited by purines, including adenosine, ATP, and ATP analogs. Adenosine A<sub>1</sub> receptors are known to mediate at least part of the response to adenine nucleotides, either because adenine nucleotides activate A<sub>1</sub> receptors directly, or activate them secondarily upon the nucleotides' conversion to adenosine. In the present studies, the inhibitory effects of adenosine, ATP, the purportedly stable ATP analog adenosine-5'-O-(3-thio)triphosphate (ATP<sub>γ</sub>S), and cyclic AMP were examined in mice with a null mutation in the adenosine A<sub>1</sub> receptor gene. ATP<sub>γ</sub>S displaced the binding of A<sub>1</sub>-selective ligands to intact brain sections and brain homogenates from adenosine A<sub>1</sub> receptor wild-type animals. In homogenates, but not in intact brain sections, this displacement

was abolished by adenosine deaminase. In hippocampal slices from wild-type mice, purines abolished synaptic responses, but slices from mice lacking functional A<sub>1</sub> receptors showed no synaptic modulation by adenosine, ATP, cAMP, or ATP<sub>γ</sub>S. In slices from heterozygous mice the dose-response curve for both adenosine and ATP was shifted to the right. In all cases, inhibition of synaptic responses by purines could be blocked by prior treatment with the competitive adenosine A<sub>1</sub> receptor antagonist 8-cyclopentyltheophylline. Taken together, these results show that even supposedly stable adenine nucleotides are rapidly converted to adenosine at sites close to the A<sub>1</sub> receptor, and that inhibition of synaptic transmission by purine nucleotides is mediated exclusively by A<sub>1</sub> receptors.

ATP released during nerve activity can regulate transmitter release (for review, see Cunha and Ribeiro, 2000). Both excitatory and inhibitory effects have been suggested in peripheral nerves, in ganglia, and in the brain (von Kugelgen et al., 1993, 1994; Boehm, 1999; O'Kane and Stone, 2000; Smith et al., 2001). For example, hippocampal synaptic transmission is inhibited not only by adenosine but also by ATP, cAMP, and numerous cAMP and ATP analogs (Dunwiddie and Hoffer, 1980; Lee et al., 1981). The inhibitory effects of adenosine are mediated via the adenosine A<sub>1</sub> receptor (A<sub>1</sub>R) (Johansson et al., 2001), but the identity of the receptor(s) by which nucleotides exert their effects on transmission is more difficult to ascertain. It is clear that ATP and other adenine nucleotides are very rapidly broken down in hippocampus, and that adenosine is one of the products formed (Dunwiddie

et al., 1997). Hence, a major question is whether ATP and other adenine nucleotides act at receptors other than presynaptic A<sub>1</sub>Rs to inhibit transmitter release.

Several lines of evidence suggest that nucleotides per se can inhibit transmission and that conversion to adenosine is not required. First, many ATP analogs, generally considered metabolically stable, are at least as potent as ATP in terms of inhibiting synaptic potentials (Lee et al., 1981; von Kugelgen et al., 1992; Cunha et al., 1998; Mendoza-Fernandez et al., 2000; Smith et al., 2001). Second, incubation of brain slices with adenosine deaminase (ADA), which is expected to convert adenosine formed from the metabolic conversion of nucleotides into inosine (relatively inactive at A<sub>1</sub>Rs) (Fredholm et al., 2001), does not completely inhibit responses to ATP and ATP analogs, suggesting that there may be a component of the ATP response that is not mediated via adenosine (Cunha et al., 1998; Mendoza-Fernandez et al., 2000). Similarly, inhibitors of 5'-nucleotidase, the extracellular enzyme required to convert 5'-AMP to adenosine, have limited effectiveness in blocking nucleotide responses. Because of these observations and additional experimental evidence, the hypothesis has been advanced that there may be a nucleotide

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**ABBREVIATIONS:** A<sub>1</sub>R, adenosine A<sub>1</sub> receptor; ADA, adenosine deaminase; CPT, 8-cyclopentyl-theophylline; ATP<sub>γ</sub>S, adenosine-5'-O-(3-thio)triphosphate; CHA, N<sup>6</sup>-cyclohexyladenosine; 2-CADO, 2-chloroadenosine; DPCPX, 1,3-dipropyl-8-cyclopentyl xanthine; R-PIA, R-N<sup>6</sup>-phenylisopropyl adenosine; fEPSP, field excitatory postsynaptic potential; EPSP, excitatory postsynaptic potential.

receptor that inhibits neurotransmitter release, is activated directly by ATP and ATP analogs, and is insensitive to most ATP receptor (P2) antagonists, but is sensitive to purportedly selective A<sub>1</sub>R antagonists such as 8-cyclopentyl-theophylline (CPT) (Shinozuka et al., 1988; Smith et al., 1997; Mendoza-Fernandez et al., 2000).

We have examined the actions of adenosine and adenine nucleotides in mice with a null mutation in the A<sub>1</sub>R gene, which results in a complete loss of functional A<sub>1</sub>Rs in the central nervous system (Johansson et al., 2001). Our experiments demonstrate that in mice lacking the A<sub>1</sub>R, the inhibitory effects of adenosine, cAMP, ATP, and adenosine-5'-O-(3-thio)triphosphate (ATP $\gamma$ S) on synaptic transmission are completely absent, suggesting that all of these actions are mediated via conventional A<sub>1</sub>Rs.

## Materials and Methods

**Null Mutant Mice.** Mice with a null mutation in the A<sub>1</sub>R were derived as described previously (Johansson et al., 2001). Mice that were heterozygous for the null mutation were bred, and littermates with the A<sub>1</sub>R<sup>+/+</sup>, A<sub>1</sub>R<sup>-/-</sup>, and A<sub>1</sub>R<sup>-/-</sup> genotypes were used for the following experiments. Mice were genotyped either by Southern blotting (Johansson et al., 2001) or by a polymerase chain reaction-based assay that amplified products of different sizes from the wild-type and null mutant genes. Phenotypes were confirmed by ligand binding assays conducted on cortical tissue obtained from the mice used in the electrophysiological studies.

**Membrane Receptor Binding Studies.** The assay method using [<sup>3</sup>H]N<sup>6</sup>-cyclohexyladenosine ([<sup>3</sup>H]CHA; PerkinElmer Life Sciences, Boston, MA) to measure A<sub>1</sub>Rs was similar to that published previously (Mayfield et al., 1996). Crude membrane preparations were preincubated in 50 mM Tris buffer, pH 7.4, and ADA (2 U/ml; Roche Applied Science, Indianapolis, IN) for 30 min at 37°C. After washing, membranes were incubated in 50 mM Tris buffer containing 10 mM MgCl<sub>2</sub> and [<sup>3</sup>H]CHA (0.14–25 nM for saturation curves, 30 nM for single point saturation analysis, and 10 nM for competition curves) for 2 h at 25°C. Nonspecific binding was measured in the presence of 2-chloroadenosine (2-CADO; 20  $\mu$ M; Sigma/RBI, Natick, MA). Competition curves with 2-CADO and ATP $\gamma$ S (Sigma-Aldrich, St. Louis, MO) were generated in the absence and presence of ADA (2 U/ml) during the incubation. Incubations were terminated by dilution with ice-cold buffer and rapid filtration through glass fiber filters. Radioactivity was quantitated by liquid scintillation counting. Saturation and competition data were analyzed by nonlinear curve fitting using the one-site hyperbola and sigmoid dose-response (variable slope) equations, respectively (GraphPad Software, San Diego, CA). Protein was measured using the method of Bradford (1976).

**Receptor Autoradiography.** Sagittal sections of brains from A<sub>1</sub>R<sup>+/+</sup> mice (14  $\mu$ m in thickness) were cut 1.2 to 1.8 mm from the midline and put on collagen-coated microscope slides. [<sup>3</sup>H]1,3-Dipropyl-8-cyclopentyl xanthine (DPCPX; 103 Ci/mmol; PerkinElmer Life Sciences) was used as the radioligand. Slides were preincubated in 170 mM Tris-HCl, pH 7.4, containing 1 mM EDTA and 4 U/ml ADA at room temperature for 30 min. Thereafter, the slides were pre-washed twice for 10 min in Tris-HCl buffer containing 1 mM MgCl<sub>2</sub> at room temperature. Then they were incubated in the same buffer with 0.5 nM [<sup>3</sup>H]DPCPX and 4 U/ml ADA and varying concentrations of ATP analogs for 2 h at room temperature. Nonspecific binding was studied in the presence of 20  $\mu$ M *R*-N<sup>6</sup>-phenylisopropyl adenosine (*R*-PIA; Sigma/RBI). The incubation was stopped by washing twice in ice-cold Tris-HCl buffer for 5 min followed by three quick dips in ice-cold distilled water. After the incubation all slides were dried with a stream of air in a refrigerator overnight, and together with calibrated standards, apposed to film for the indicated times.

Exposure time was 2 to 3 weeks. The autoradiograms were analyzed with an M5 Imaging Device (Imaging Research, St. Catharines, ON, Canada). Optical densities were converted to binding density (femtomoles per milligram of gray matter) using the plastic standards and the specific activity of the radioligands. Results and dose-response curves were analyzed with GraphPad Prism.

**Purine Metabolism.** Cerebral cortical and hippocampal tissue from A<sub>1</sub>R<sup>+/+</sup> and A<sub>1</sub>R<sup>-/-</sup> mice was homogenized in 5 ml of Tris-buffered sucrose. After removal of nuclei and debris by centrifugation, ATP was added to a final concentration of 30  $\mu$ M. Using high-performance liquid chromatography, the levels of ATP, ADP, AMP, adenosine, and inosine were determined at 5-min intervals during a 30-min incubation at 30°C.

**Electrophysiology.** Hippocampal slices (400  $\mu$ m) were prepared from 4- to 24-week-old mice and studied using conventional extracellular and whole-cell recording techniques (Dunwiddie and Diao, 1994; Poelchen et al., 2000). Brain slices were maintained at a constant temperature of 31–33°C in a submersion chamber and constantly superfused (2 ml/min) with gassed (95% O<sub>2</sub>, 5% CO<sub>2</sub>) artificial cerebrospinal fluid containing 126 mM NaCl, 3 mM KCl, 1.5 mM MgCl<sub>2</sub>, 2.4 mM CaCl<sub>2</sub>, 1.2 mM NaH<sub>2</sub>PO<sub>4</sub>, 11 mM glucose, and 26 mM NaHCO<sub>3</sub> (Sigma-Aldrich). For recordings of field excitatory postsynaptic potential (fEPSP) responses, the recording electrode was placed in stratum radiatum of the CA1 region and the stimulation electrode in stratum radiatum near the border of the CA1 and CA2 regions; stimuli were delivered at 15-s intervals. EPSPs were recorded from CA1 neurons using the whole-cell patch-clamp technique. Patch recording electrodes were filled with a solution containing 125 mM potassium-gluconate, 5 mM KCl, 10 mM HEPES, 0.1 mM CaCl<sub>2</sub>, 1 mM potassium-EGTA, 2 mM MgCl<sub>2</sub>, 2 mM magnesium-ATP, and 0.2 mM Tris-GTP (Sigma-Aldrich). Series resistances ranged from 10 to 41 M $\Omega$  (average 30  $\pm$  1.5 M $\Omega$ ).

For delivery of drug solutions, stock solutions of adenosine, ATP, cAMP, ATP $\gamma$ S (Sigma-Aldrich), or 8-cyclopentyltheophylline (CPT; Sigma/RBI) were prepared at 50 to 200 times the desired final concentration of drug, and a calibrated syringe pump (Razel, Stamford, CT) was used to add the drugs directly to the superfusion system. Concentration-response curves were made by superfusing slices with incrementally higher concentrations of drugs until a near-maximal inhibition of the EPSP was observed, because in no case was there any evidence of desensitization of the response. EC<sub>50</sub> values and Hill slopes for the concentration-response curves were determined using the InPlot program (GraphPad Software), with the  $E_{\min}$  constrained to 0 and the  $E_{\max}$  to 100%.

## Results

**ATP and ATP Analogs Displace Binding of Selective A<sub>1</sub>R Ligands.** In the present experiments, agonist ([<sup>3</sup>H]CHA) binding was measured in the cerebral cortices of the same mice used in the electrophysiological experiments. As with an antagonist radioligand ([<sup>3</sup>H]DPCPX; Johansson et al., 2001), the  $B_{\max}$  in the A<sub>1</sub>R<sup>-/-</sup> mice was half of that in the A<sub>1</sub>R<sup>+/+</sup> mice (52%), and there was virtually no specific binding in the A<sub>1</sub>R<sup>-/-</sup> animals (4.5% of A<sub>1</sub>R<sup>+/+</sup> mice; Table 1). Thus, these experiments confirm that the null mutation results in a complete loss of A<sub>1</sub>Rs and that neither [<sup>3</sup>H]DPCPX nor [<sup>3</sup>H]CHA bind with high-affinity binding to other non-A<sub>1</sub>R-related binding sites in brain.

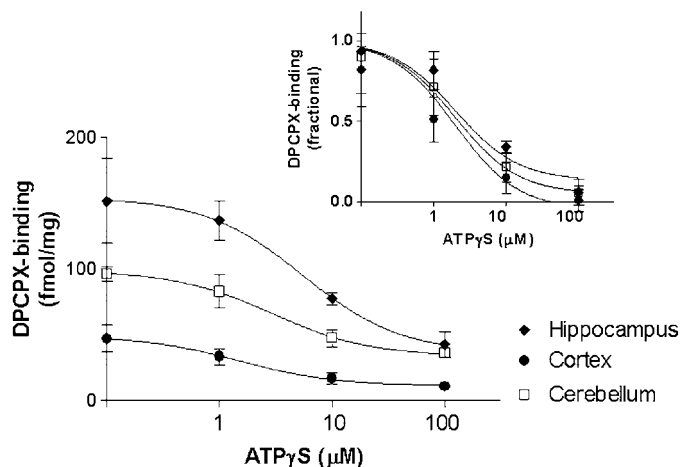
Despite the demonstrated specificity of [<sup>3</sup>H]DPCPX and [<sup>3</sup>H]CHA binding to A<sub>1</sub>Rs, we observed that a purportedly stable ATP analog, ATP $\gamma$ S, was able to displace their binding. In sections of hippocampus, cerebellum, and cortex from A<sub>1</sub>R<sup>+/+</sup> mice, ATP $\gamma$ S was a potent inhibitor (IC<sub>50</sub> values of 5  $\pm$  1.4, 3  $\pm$  1.5, and 2  $\pm$  1.4  $\mu$ M, respectively), even in the presence of 4 U/ml ADA (Fig. 1). Analogous data were obtained using 5'-adenylymidodiphosphate (data not shown). These results indicate that these ATP analogs can interact with A<sub>1</sub>Rs and that this is not due to the ATP analogs being contaminated by adenosine.

TABLE 1

Binding of [<sup>3</sup>H]CHA to mouse cortical membranesBinding was determined either from full saturation curves ( $K_d$  and  $B_{max}$ ), or by measuring specific binding with a saturating concentration of [<sup>3</sup>H]CHA (~30 nM). Both approaches gave comparable values for the total number of A<sub>1</sub> binding sites. Mean values ± S.E.M. Number of animals is indicated in parentheses.

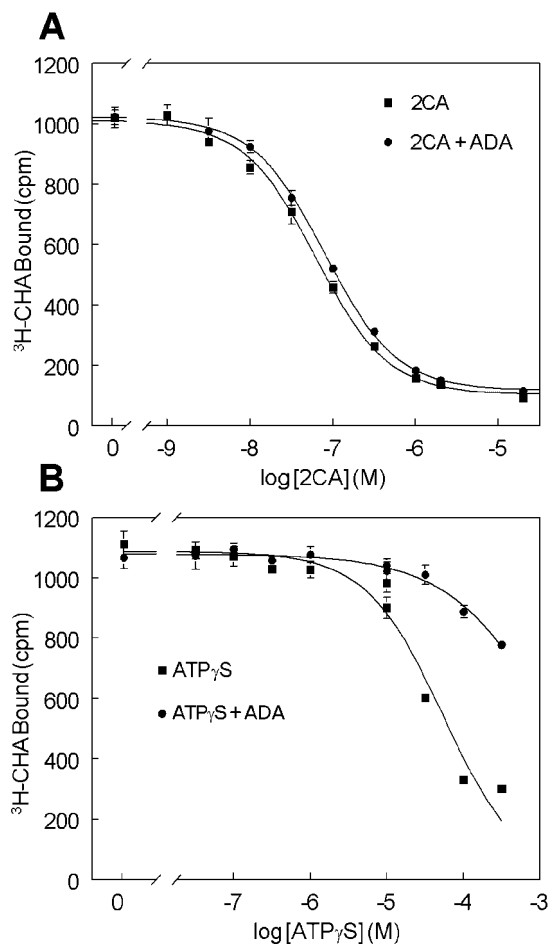
Genotype	A <sub>1</sub> R <sup>+/+</sup>	A <sub>1</sub> R <sup>+/-</sup>	A <sub>1</sub> R <sup>-/-</sup>
$K_d$ (nM)	0.56 ± 0.02 (7)	0.60 ± 0.06 (5)	N.D.
$B_{max}$ (CHA) (fmol/mg protein)	818 ± 25.6 (7)	429 ± 35.5 (5)	N.D.
Specific binding (fmol/mg protein)	858 ± 27.6 (18)	453 ± 28.2 (13)	38.7 ± 5.19 (11)

N.D., not determined.



**Fig. 1.** Displacement of [<sup>3</sup>H]DPCPX by ATP $\gamma$ S in intact brain sections. Binding experiments were carried out in the presence of 4 U/ml adenosine deaminase. Despite this, ATP $\gamma$ S significantly displaced [<sup>3</sup>H]DPCPX (0.5 nM) from its binding sites. These experiments were carried out in sections from A<sub>1</sub>R<sup>+/+</sup> mice; A<sub>1</sub>R<sup>-/-</sup> mice show essentially no DPCPX binding (Johansson et al., 2001). The calculated IC<sub>50</sub> values were hippocampus, 5 ± 1.4  $\mu$ M; cerebellum, 3 ± 1.5  $\mu$ M; and cortex, 2 ± 1.4  $\mu$ M (mean values ± S.E.M.;  $n$  = 6; calculated on nontransformed data). Inset, to illustrate the relative displacement in different brain regions, the same data were normalized by subtracting maximal displacement and setting maximal binding to 1.

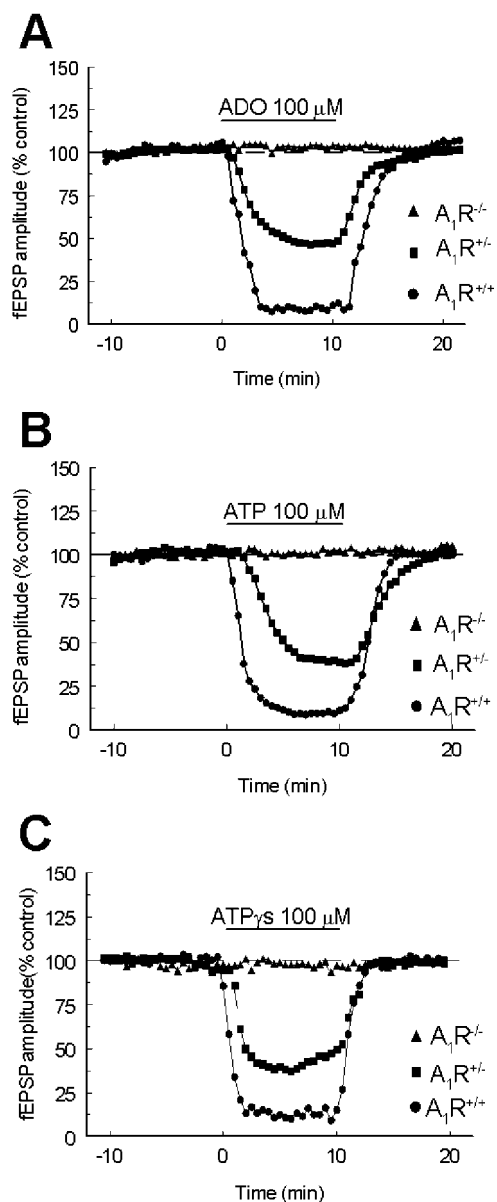
Similar experiments were carried out to determine the ability of a stable adenosine analog, 2-CADO, and ATP $\gamma$ S to displace [<sup>3</sup>H]CHA from high-affinity binding sites. Because of limited tissue availability, these experiments were conducted in membranes prepared from rat cortex; previous studies have shown that the rat and mouse receptors have very similar pharmacological properties (Maemoto et al., 1997). When A<sub>1</sub>R<sup>+/+</sup> mouse and Sprague-Dawley rat cortical membranes were assayed with [<sup>3</sup>H]CHA in parallel, they showed nearly identical  $K_d$  (1.12 ± 0.29 versus 0.97 ± 0.12 nM) and  $B_{max}$  (724 ± 28.5 versus 726 ± 7.99 fmol/mg protein) values in mice and in rats, respectively. All membranes were pretreated with ADA to eliminate endogenous adenosine, and subsequently the competition curves were generated either in the presence or absence of ADA. The ability of 2-CADO to displace [<sup>3</sup>H]CHA binding was unaffected by the presence or absence of ADA in the assay (Fig. 2A). In the absence of ADA, ATP $\gamma$ S displaced [<sup>3</sup>H]CHA significantly at concentrations as low as 10  $\mu$ M, but in the presence of ADA its apparent potency was markedly reduced (Fig. 2B). Interestingly, the potency of ATP $\gamma$ S to interact with A<sub>1</sub> receptors in the brain section in the presence of ADA was as high as that in the homogenate in the absence of ADA. Together, these results suggest that even this “stable” adenosine nucleotide is converted to adenosine, which then interacts with the A<sub>1</sub>Rs. However, in a preparation with a more intact cellular structure, such as a brain section or brain slice, ADA has a limited capacity to eliminate adenosine formed locally by ATP hydrolysis, perhaps due to limited access, and the effect of an adenosine nucleotide is profound even in the presence of ADA. These results thus agree with initial observations by Williams and Braunwalder (1986) and the more recent suggestion by Cunha et al. (1998) that in an intact



**Fig. 2.** Displacement of [<sup>3</sup>H]CHA by 2-CADO and ATP $\gamma$ S in rat cortical membranes in the absence and presence of ADA. A, presence of ADA had essentially no effect upon the ability of 2-CADO to displace [<sup>3</sup>H]CHA binding (IC<sub>50</sub> values were 62.5 ± 7.14 and 80.3 ± 8.88 nM in the absence and presence of 2 U/ml ADA, respectively). B, in the absence of ADA, the IC<sub>50</sub> for ATP $\gamma$ S was 32.0 ± 3.46  $\mu$ M, whereas in the presence of this enzyme, an IC<sub>50</sub> could not be determined, but was well in excess of 300  $\mu$ M, the highest concentration tested (mean values ± S.E.M.;  $n$  = 3 for each data set).

hippocampal slice, adenosine nucleotides can be hydrolyzed to adenosine in a local compartment that is not in equilibrium with the incubation medium. This is particularly relevant, because such preparations are used routinely in electrophysiological studies of presynaptic regulation and synaptic function.

**Electrophysiological Actions of Adenosine, ATP, and ATP $\gamma$ S Are Eliminated in A<sub>1</sub>R Knockout Mice.** The inhibitory effects of adenosine on synaptically evoked fEPSP responses were examined in A<sub>1</sub>R<sup>+/+</sup>, A<sub>1</sub>R<sup>-/-</sup>, and A<sub>1</sub>R<sup>+/-</sup> mice. As we have reported previously (Johansson et al., 2001), adenosine had an inhibitory effect on fEPSPs in hippocampal slices from A<sub>1</sub>R<sup>+/+</sup> and A<sub>1</sub>R<sup>-/-</sup> mice but had no effect on responses in A<sub>1</sub>R<sup>+/-</sup> slices. The time course of inhibition was similar in A<sub>1</sub>R<sup>+/+</sup> and A<sub>1</sub>R<sup>-/-</sup> slices (Fig. 3A). The



**Fig. 3.** Time course and individual examples of responses to adenosine, ATP, and ATP $\gamma$ S. A, response to adenosine is shown for individual slices obtained from A<sub>1</sub>R<sup>+/+</sup>, A<sub>1</sub>R<sup>-/-</sup>, and A<sub>1</sub>R<sup>+/-</sup> mice that were tested with an identical experimental protocol (10-min superfusion with 100  $\mu$ M adenosine). The response to 100  $\mu$ M adenosine showed a very similar time course in slices from the A<sub>1</sub>R<sup>+/+</sup> and A<sub>1</sub>R<sup>-/-</sup> animals, but the maximal response in the A<sub>1</sub>R<sup>-/-</sup> slice was approximately half that seen in the A<sub>1</sub>R<sup>+/+</sup> slice. B and C, effect of 100  $\mu$ M ATP and ATP $\gamma$ S, respectively, on hippocampal slices obtained from mice of each of the three genotypes. Both the magnitude and the time course of the responses were similar to what was observed with adenosine.

EC<sub>50</sub> for inhibition of the fEPSP response by adenosine was significantly greater in the A<sub>1</sub>R<sup>-/-</sup> slices ( $72 \pm 3.0 \mu$ M) than in the A<sub>1</sub>R<sup>+/+</sup> slices ( $40 \pm 1.5 \mu$ M,  $p < 0.0001$ ; Table 2). There was no difference in the  $E_{max}$  values between the A<sub>1</sub>R<sup>+/+</sup> and A<sub>1</sub>R<sup>-/-</sup> slices, which suggests that there are spare receptors for this inhibitory response in the wild-type mice. As reported for rat hippocampal slices, whenever adenosine depressed the fEPSP response there was a reduction in both the peak amplitude and the slope of the falling phase of the fEPSP, but there was no change in the presynaptic fiber volley.

The effects of ATP were also examined in slices from all three genotypes. The time course of inhibition by ATP was similar in A<sub>1</sub>R<sup>+/+</sup> and A<sub>1</sub>R<sup>-/-</sup> slices, even though the maximum depression

TABLE 2

Effects of adenosine and adenine nucleotides on fEPSP responses

Dose-response curves were obtained for adenosine and ATP in A<sub>1</sub>R<sup>+/+</sup> and A<sub>1</sub>R<sup>+/-</sup> mice (Fig. 4). The EC<sub>50</sub> and the Hill slope were significantly higher for ATP compared with adenosine, both in A<sub>1</sub>R<sup>+/+</sup> and A<sub>1</sub>R<sup>-/-</sup> slices. In addition, the EC<sub>50</sub> and Hill slope for both adenosine and ATP were higher in the A<sub>1</sub>R<sup>+/-</sup> compared with A<sub>1</sub>R<sup>+/+</sup> slices.

Genotype	A <sub>1</sub> R <sup>+/+</sup>		A <sub>1</sub> R <sup>+/-</sup>	
	Adenosine	ATP	Adenosine	ATP
EC <sub>50</sub>	40 ± 1.5	90 ± 1.5 <sup>*a</sup>	72 ± 3.0 <sup>*b</sup>	130 ± 4.6 <sup>*a,b</sup>
Hill slope	1.1 ± 0.45	1.8 ± 0.22 <sup>*a</sup>	1.5 ± 0.17 <sup>*b</sup>	2.3 ± 0.28 <sup>*a,b</sup>

<sup>\*</sup>  $p < 0.05$ .

<sup>a</sup> ATP versus adenosine.

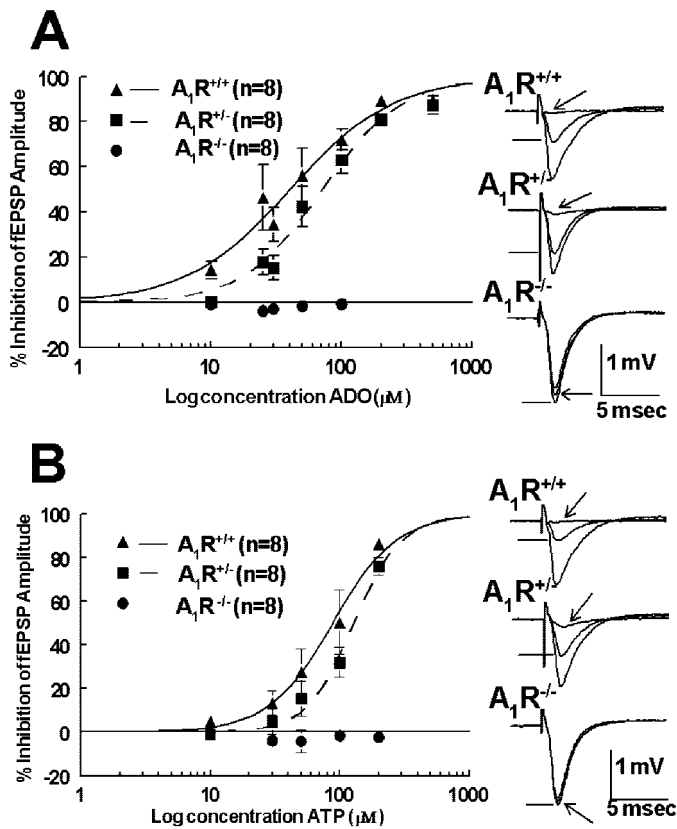
<sup>b</sup> A<sub>1</sub>R<sup>+/-</sup> vs. A<sub>1</sub>R<sup>+/+</sup>.

required slightly more time in the A<sub>1</sub>R<sup>-/-</sup> slices (Fig. 3B). Similar to adenosine, the EC<sub>50</sub> value for ATP was significantly higher in A<sub>1</sub>R<sup>-/-</sup> than A<sub>1</sub>R<sup>+/+</sup> slices (Table 2). In addition, the EC<sub>50</sub> value for ATP was higher than that for adenosine (Table 2). ATP had no effect on synaptic transmission in A<sub>1</sub>R<sup>-/-</sup> slices (Fig. 3B).

Because ectonucleotidases are known to convert ATP to adenosine in brain (Zimmermann, 1996), and rapid conversion has been demonstrated in the rat hippocampus (Dunwiddie et al., 1997), the lack of ATP effects in the A<sub>1</sub>R<sup>-/-</sup> slices might be attributable to rapid breakdown of ATP. For this reason, many studies of nucleotide receptors focus on metabolically stable ATP analogs such as ATP $\gamma$ S, which has been shown to be much more stable than ATP when incubated with hippocampal slices or synaptosomes (Cunha et al., 1998). Therefore, we characterized the actions of ATP $\gamma$ S on fEPSP responses, to determine whether there were nucleotide actions that were not observed with application of ATP because of its rapid conversion to adenosine. When slices from A<sub>1</sub>R<sup>+/+</sup> mice were superfused with 100  $\mu$ M ATP $\gamma$ S, the fEPSP response was inhibited in a manner identical to that observed with ATP or adenosine (Fig. 3C). In the A<sub>1</sub>R<sup>-/-</sup> slices, similar to ATP and adenosine, the response to ATP $\gamma$ S was reduced. Most significantly, like ATP and adenosine, 100  $\mu$ M ATP $\gamma$ S had no detectable effect on fEPSP responses in slices from A<sub>1</sub>R<sup>-/-</sup> mice (Fig. 3C). The comparable time course seen with all three compounds (Fig. 3) suggests that if the purine nucleotides are being converted to adenosine within the slice, this must be occurring relatively rapidly with respect to the time required for the adenosine/ATP to diffuse into the slice.

Complete concentration-response curves were obtained for adenosine and ATP. In both the A<sub>1</sub>R<sup>+/+</sup> and A<sub>1</sub>R<sup>-/-</sup> mouse hippocampal slices, high concentrations of both adenosine (Fig. 4A) and ATP (Fig. 4B) were able to completely inhibit the fEPSP response. However, in the A<sub>1</sub>R<sup>-/-</sup> slices, neither adenosine nor ATP had a significant effect on the response waveform at concentrations up to 200  $\mu$ M (Fig. 4).

Previous studies in hippocampal brain slices from rat have demonstrated that cAMP and ATP both inhibit the fEPSP response in a manner that is similar to adenosine, although ATP is slightly less potent, and cAMP is significantly more potent than adenosine (Dunwiddie and Hoffer, 1980; Lee et al., 1981; Dunwiddie et al., 1997). These effects have generally been ascribed to metabolism of these nucleotides to adenosine by ectophosphodiesterases and ectonucleotidases, because these actions are antagonized by adenosine receptor antagonists (Dunwiddie et al., 1997). However, there is considerable variation between species with respect to some of the nucleotidases, and the mouse in particular shows considerably less 5'-nucleotidase activity in the CA1 region of the hippocampus than does the rat (Lee et al., 1986). Therefore, the effect of cAMP was also determined in hippocampal slices from all three types of mice. As with ATP and ATP $\gamma$ S, superfusion of slices with cAMP inhibited the fEPSP responses. Complete concentration-response curves were not obtained for cAMP, but the magnitude of the inhibition produced by 50  $\mu$ M cAMP was quite comparable with the inhibition induced by 50  $\mu$ M adenosine, ATP, or ATP $\gamma$ S in both the A<sub>1</sub>R<sup>+/+</sup> and A<sub>1</sub>R<sup>-/-</sup> hippocampal slices (Fig. 5A). As with adenosine and the other ade-

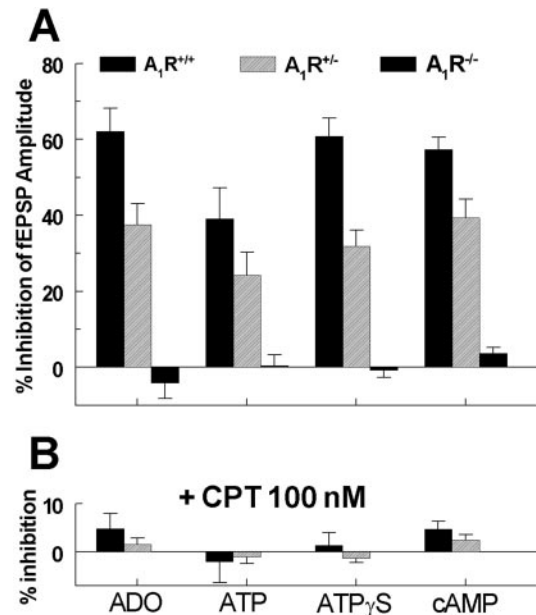


**Fig. 4.** Adenosine and ATP concentration-response curves and synaptic responses. **A**, adenosine-mediated depression of fEPSP amplitude was examined in hippocampal slices from  $A_1R^{+/+}$ ,  $A_1R^{+/-}$ , and  $A_1R^{-/-}$  mice. The curves shown are the best fits to the raw data for each line of mice;  $E_{\text{max}}$  was constrained to 100% in each case. As has been reported previously, there was no detectable effect of adenosine in the  $A_1R^{-/-}$  mice. Recordings of fEPSP responses evoked from hippocampal slices of each genotype are shown on the right. The heavier trace is the control response, the horizontal line denotes the response in the presence of 100  $\mu\text{M}$  adenosine, and the arrow the response in the presence of 200  $\mu\text{M}$  adenosine. **B**, ATP-mediated depression of fEPSP amplitude was examined in all three genotypes as in **A**. The curves are the best fits to the raw data with  $E_{\text{max}}$  constrained to 100%. As with adenosine, ATP was significantly more potent in the  $A_1R^{+/+}$  than in the  $A_1R^{-/-}$  slices, and there was no effect in the  $A_1R^{-/-}$  slices. Averaged fEPSP responses are shown on the right. As in **A**, the heavier trace in each set of records is the control response, the horizontal line denotes the response in the presence of 100  $\mu\text{M}$  ATP, and the arrow the response in the presence of 200  $\mu\text{M}$  ATP. Scale bars apply to synaptic responses.

nine nucleotides, cAMP had no detectable effect on responses evoked in the  $A_1R^{-/-}$  slices (Fig. 5A).

In addition to comparing the effects of nucleotides in the intact slice, we assayed ATP hydrolysis, adenosine elimination, and inosine formation in homogenates of hippocampus and cortex of wild-type and knockout mice. There was no difference in either the rate of ATP hydrolysis (for example, after 15 min, ATP levels were reduced from 30 to  $13.4 \pm 0.9 \mu\text{M}$  in  $A_1R^{+/+}$  cortex and from 30 to  $13.2 \pm 0.5 \mu\text{M}$  in  $A_1R^{-/-}$  cortex) or the rate of AMP accumulation (data not shown). Additionally, there was no difference between the genotypes in the rate of adenosine elimination (adenosine levels decreased from  $3.7 \pm 0.2$  to  $2.3 \pm 0.4 \mu\text{M}$  in  $A_1R^{+/+}$  cortex and from  $3.5 \pm 0.5$  to  $2.2 \pm 0.3 \mu\text{M}$  in  $A_1R^{-/-}$  cortex during 15 min) or inosine formation (data not shown). Furthermore, results were similar in hippocampus and in cortex (data not shown).

Finally, to compare between species, we measured the effects of 100  $\mu\text{M}$  ATP $\gamma$ S on the fEPSP recorded from wild-type mouse versus rat hippocampal slices. There was no difference in the amount of



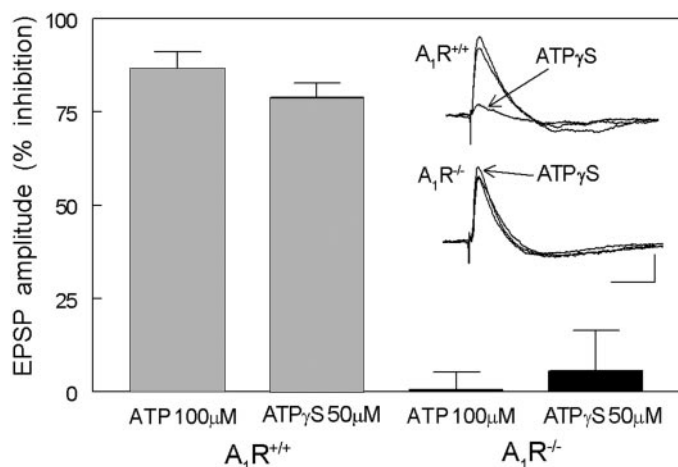
**Fig. 5.** Comparison of the effects of adenosine and adenine nucleotides. Slices from  $A_1R^{+/+}$ ,  $A_1R^{+/-}$ , and  $A_1R^{-/-}$  mice were superfused with 50  $\mu\text{M}$  adenosine (ADO), ATP, ATP $\gamma$ S, or cAMP, either under control conditions (**A**) or in the presence of 100 nM CPT (**B**; pretreated with CPT for 30 min before agonist superfusion). **A**, in all cases of purine superfusion, the response in the  $A_1R^{+/+}$  slices was larger than in the  $A_1R^{-/-}$  slices, and there was no significant response in the  $A_1R^{-/-}$  slices. **B**, pretreatment with CPT, an  $A_1R$  antagonist, abolished the effects of purine application in  $A_1R^{+/+}$  and  $A_1R^{-/-}$  slices. Because there was no significant effect of any agonist in slices from the  $A_1R^{-/-}$  mice, the effects of CPT were not examined in these slices. Previous studies have shown that  $A_1R$  antagonists have no effect on responses from  $A_1R^{-/-}$  slices (Johansson et al., 2001).

inhibition observed in slices obtained from the two species (mouse,  $-79.4 \pm 6.0\%$ ; rat,  $-89.1 \pm 1.4\%$ ;  $n = 6$  for each; N.S.)

The results obtained with the  $A_1R^{-/-}$  slices suggest that the responses to ATP $\gamma$ S are mediated by  $A_1R$ s. This was confirmed pharmacologically by determining the sensitivity of these responses to CPT, a selective  $A_1R$  antagonist. As has been reported previously in rat, responses in both the  $A_1R^{+/+}$  and  $A_1R^{-/-}$  slices to 50  $\mu\text{M}$  adenosine, ATP, ATP $\gamma$ S (Mendoza-Fernandez et al., 2000), and cAMP were completely antagonized by prior superfusion with 100 nM CPT (Fig. 5B).

A previous report suggesting that nucleotide effects on hippocampal synaptic transmission might be mediated via other purine receptors (Mendoza-Fernandez et al., 2000) used intracellular recording of EPSPs to characterize modulation of transmission, rather than measurements of synaptic field potentials. Although fEPSPs and intracellularly recorded EPSPs are generally affected in the same ways by pharmacological agents, this is not always the case (Dunwiddie et al., 1980; Robinson and Deadwyler, 1981; Valentino et al., 1982), so parallel experiments were conducted using whole-cell patch recordings from mouse hippocampal slices to confirm the previous observations. As was observed with extracellular field potentials, in these experiments both ATP and ATP $\gamma$ S inhibited the intracellularly recorded EPSPs, although the intracellularly recorded responses seemed to be slightly more sensitive in this regard (Fig. 6). Moreover, as seen in the field potentials, the effects of both nucleotides are essentially lost in  $A_1R^{-/-}$  slices, clearly implicating the involvement of  $A_1R$ s in this response.

In addition to presynaptic  $A_1R$ s, CA1 pyramidal neurons also have postsynaptic  $A_1R$ s that activate a G protein-coupled inwardly rectifying  $K^+$  conductance that hyperpolarizes these cells (Greene and Haas, 1985), and we have previously reported that adenosine induces an outward (i.e., hyperpolarizing) conductance in pyramidal



**Fig. 6.** Effects of adenine nucleotides on intracellularly recorded EPSPs. Hippocampal slices were superfused with the indicated concentrations of nucleotides while EPSPs were recorded using whole-cell patch electrodes. EPSPs were profoundly inhibited by ATP and ATP $\gamma$ S in the  $A_1R^{+/+}$  animals, but not in cells from slices from  $A_1R^{-/-}$  animals. Each bar is the mean  $\pm$  S.E.M. inhibition in response to agonist in three to six cells recorded from animals with the indicated genotype. The inset shows individual examples of EPSPs evoked in a  $A_1R^{+/+}$  and a  $A_1R^{-/-}$  CA1 pyramidal neuron under control conditions, during superfusion with 50  $\mu$ M ATP $\gamma$ S (indicated by the arrows), and after washout (calibration bars indicate 2 mV and 50 ms).

neurons from  $A_1R^{+/+}$  mice that is absent in  $A_1R^{-/-}$  mice (Johansson et al., 2001). In the present studies, we have observed that ATP induced a small but statistically significant hyperpolarizing effect on the resting membrane potential of CA1 neurons in  $A_1R^{+/+}$  mice (hyperpolarized by  $4.2 \pm 1.2$  mV;  $p < 0.05$ ,  $n = 4$ ) that was not observed in  $A_1R^{-/-}$  mice. In contrast, ATP $\gamma$ S depolarized  $A_1R^{+/+}$  CA1 neurons (depolarized by  $7.5 \pm 1.5$  mV;  $p < 0.05$ ,  $n = 3$ ), and this effect seemed to be present in the  $A_1R^{-/-}$  mice (depolarized by  $8.9 \pm 4.8$  mV; N.S.,  $n = 3$ ).

## Discussion

Using brain slices and tissue homogenates from mice bred to be  $+/+$ ,  $-/-$ , or  $-/-$  for the  $A_1R$ , we clarify several issues that have remained difficult to resolve with pharmacologically based experiments. We show that the two commonly used radioligands, the  $A_1R$  agonist [ $^3H$ ]CHA, and the  $A_1R$  antagonist [ $^3H$ ]DPCPX, are selective for  $A_1R$ s in concentrations commonly used in binding studies. Hence, previous suggestions that there are binding sites for [ $^3H$ ]DPCPX at some as yet poorly defined nucleotide receptor (Smith et al., 1997) were not supported. In addition, we confirmed that ATP and some purportedly stable ATP analogs do compete for  $A_1R$  ligands at their binding sites and have similar electrophysiological effects as  $A_1R$  agonists on hippocampal slices from all three types of mice.

Less is known about the effects of adenosine in the mouse compared with the rat. Nevertheless, presynaptic as well as postsynaptic effects of adenosine observed in the rat are recapitulated in the mouse (Luscher et al., 1997; Greif et al., 2000; Jarolimek et al., 2000). Herein, we report that fEPSP responses evoked by Schaffer collateral/commissural stimulation are potently inhibited by adenosine, although the  $EC_{50}$  for this response was higher than we observed previously in the rat (Dunwiddie et al., 1986; Brundage et al., 1997). Given the nearly identical  $B_{max}$  and  $K_d$  for  $A_1R$ s between the two species (Maemoto et al., 1997), this difference is most likely

due to different coupling between the receptor and modulation of transmitter release, although differences in adenosine uptake could also be involved. In  $A_1R^{-/-}$  mice, which express approximately 50% of the  $A_1R$ s seen in the wild-type mice, there was a significant increase in the  $EC_{50}$  for the inhibition of the fEPSP by adenosine, although there was no change in the maximal inhibition. Similar results (i.e., a change in  $EC_{50}$  but not in  $E_{max}$ ) have been reported in the rat when the number of  $A_1R$ s is reduced (Sebastiao et al., 2000). These data are consistent with the conclusion that there are spare receptors for the inhibition of synaptic potentials and that the reduction in the number of receptors in the  $A_1R^{-/-}$  animals results in a synapse where the same maximal inhibition can be achieved but requires occupation of a greater fraction of the receptors in the  $A_1R^{-/-}$  versus the  $A_1R^{+/+}$  animals. The ligand binding studies clearly suggest no difference in receptor affinity for agonist between the  $A_1R^{+/+}$  and the  $A_1R^{-/-}$  animals, so the changes observed are most likely related to changes in the number of receptors.

Insofar as the responses to ATP and ATP $\gamma$ S are concerned, there are three basic possibilities: 1) nucleotides bind to and directly activate  $A_1R$ s; 2) ectonucleotidases metabolize extracellular nucleotides to adenosine, which then activates  $A_1R$ s; or 3) nucleotides activate novel ATP receptors antagonized by classical  $A_1R$  antagonists such as CPT. Regarding the first possibility, the present experiments show ATP $\gamma$ S to be a very weak displacer of [ $^3H$ ]CHA binding. This agrees with results from other groups for both the  $A_1R$  (Schwabe and Trost, 1980; Ragazzi et al., 1991) as well as the  $A_{2A}$  receptor (Piroton and Boeynaems, 1993). In contrast, the second possibility, metabolism of nucleotides by ectonucleotidases, is supported both by our studies as well as those of others. For example, in brain membranes sufficient adenosine can be formed from stable ATP analogs to interfere with the binding of high-affinity ligands (Figs. 1 and 2; Piroton and Boeynaems, 1993). In homogenates of rat brain the displacement by ATP $\gamma$ S was virtually eliminated by incubation with the enzyme adenosine deaminase. This suggests that these ATP analogs interact with  $A_1$  receptors because they are converted to adenosine. It may be argued that the addition of exogenous ATP could in some way alter the breakdown of endogenous adenosine and that this would explain our results. However, this would not explain the complete absence of any response in the  $A_1R^{-/-}$ . This cannot be addressed readily in the intact slice, but when using brain homogenates the rate of adenosine elimination in the presence of ATP was not different between wild-type and knockout tissues. The rate of ATP hydrolysis was also indistinguishable.

The third possibility, nucleotides activate novel ATP receptors, is disproved by the finding that the inhibition of fEPSP responses by adenosine, ATP, ATP $\gamma$ S, and cAMP was reduced in the  $A_1R^{-/-}$  and completely lost in the  $A_1R^{-/-}$  slices. The most straightforward conclusion based upon the present results is that in the hippocampus these nucleotides inhibit excitatory neurotransmission via  $A_1R$ s, and most likely following their conversion to adenosine by ectonucleotidases.

Previously, Cunha et al. (1998) suggested that nucleotide hydrolysis may occur at a site quite close to the adenosine receptors themselves. Our data showing that ADA does not prevent adenine nucleotides from interacting with  $A_1$  receptors in brain sections (even though ADA prevents binding in homogenates) is entirely compatible with localized genera-

tion of adenosine from adenine nucleotides. The observation that both ectonucleotidases and A<sub>1</sub>Rs are localized to caveolae in non-neural tissues (Kittel and Bacsy, 1994; Lasley et al., 2000) suggests there may be mechanisms by which these proteins associate in similar domains in cellular membranes. Thus, using the terminology of Cunha et al. (1998), adenosine that is produced by the nucleotidases is "channeled" to the adenosine receptors. Such localized adenosine production accounts for the fact that significant adenosine-mediated inhibition can be achieved even when there is very limited conversion of stable nucleotides to adenosine, as determined using biochemical methods (Cunha et al., 1998). Thus, an active nucleotidase might generate substantial local concentrations of adenosine from an adenine nucleotide, whereas converting relatively little of the nucleotide to adenosine in a quantitative sense.

Even though ATP and ATP $\gamma$ S had very similar effects on presynaptic release of transmitter, this was not the case at postsynaptic A<sub>1</sub>Rs where ATP hyperpolarized and ATP $\gamma$ S depolarized the membrane potential. One explanation for this discrepancy between the presynaptic and postsynaptic A<sub>1</sub>Rs could be that ectonucleotidases are located in proximity to the presynaptic but not postsynaptic receptors, and thus the local concentration of adenosine at the postsynaptic site is insufficient to activate the A<sub>1</sub>Rs. Alternatively, the depolarizing response to ATP $\gamma$ S may simply have occluded a hyperpolarizing response to this nucleotide.

The findings of Mendoza-Fernandez et al. (2000) are somewhat difficult to reconcile with the conclusions of the present study. In particular, their observation that ATP responses were substantially more sensitive to pertussis toxin than adenosine responses suggests there may be additional complexities involved in the actions of ATP. Colocalization and coimmunoprecipitation of A<sub>1</sub>R with P2Y<sub>1</sub> receptors after their coexpression in human embryonic kidney cells was recently reported (Yoshioka et al., 2001). Further experiments are needed to determine whether functional P2Y<sub>1</sub>/A<sub>1</sub> receptors exist in hippocampus and whether they are peculiarly sensitive to pertussis toxin. Another possibility is that the nucleotidase is in some way dependent on a G protein pathway to be fully competent, and additional more or less far-fetched explanations can be devised. We favor the view that adenosine in the extracellular buffer reaches a somewhat different population of A<sub>1</sub>Rs than adenosine channeled to the receptors from ATP or its analogs. Given that there are many spare receptors, equivalent responses may be produced via slightly different entities that may differ in their ability to be reached by pertussis toxin.

The apparent Hill slopes for the adenosine and ATP concentration-response curves were significantly different. Although this might be taken as evidence that different receptors mediate the two responses, it is perhaps not surprising because the concentrations quantified are bath, not tissue concentrations of drug. Previous work from our laboratory has demonstrated that upon superfusion with an EC<sub>50</sub> concentration of adenosine (approximately 25  $\mu$ M in rat), approximately 97% of the adenosine in the extracellular buffer is taken up by cells or metabolized before it reaches receptor sites (Dunwiddie and Diao, 1994). If ATP must be converted to adenosine to be effective at A<sub>1</sub>Rs then this concentration additionally depends on its conversion by nucleotidases. Thus, one would not necessarily expect the concentration of

adenosine or ATP in the vicinity of the receptors to be linearly related to the bath concentration, because both metabolism and transport are saturable processes.

In summary, the results of the present experiments suggest that bath superfusion with adenine nucleotides, including ATP and ATP $\gamma$ S, leads to the activation of hippocampal adenosine A<sub>1</sub>Rs. Ligand binding studies provide direct evidence that these nucleotides themselves have low affinity for A<sub>1</sub>Rs, so it is most probable that they activate A<sub>1</sub>Rs after conversion to adenosine by ectonucleotidases, although other possibilities (e.g., activation after conversion to AMP) cannot be ruled out. In general, ATP responses have been controversial and difficult to study in the hippocampus, due to some inadequacies of available pharmacological tools and potential contamination with adenosine responses. The A<sub>1</sub>R null mutant mouse avoids a number of these issues, which are difficult to resolve in wild-type animals, and serves as a useful model to study and clarify purinergic receptor function.

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