0022-3565/96/2771-0193$03.00/0
THE JOURNAL OF PHARMACOLOGY AND EXPERIMENTAL THERAPEUTICS
Vol. 277, No. 1
Printed in U.S.A.

JPET 277:193–197, 1996

A_1, but not A_{2A}, Adenosine Receptors Modulate Electrically Stimulated [^{14}C]Acetylcholine Release from Rat Cortex[1]

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Accepted for publication December 18, 1995

ABSTRACT

Adenosine A_1 receptors are known to be widely distributed in various regions of the brain. A_{2A} receptors are enriched in the dopamine-rich areas of the brain, but are also present in rat cortex. Electrically stimulated, perfused rat cortical slices were used to examine the influence of interactions between A_1 and A_{2A} receptors on the release of acetylcholine (ACh) from cortical cholinergic nerves. The A_1-selective agonist, N^6-cyclopentyladenosine (CPA) caused a dose-dependent inhibition of ACh release, which was attenuated in the added presence of the A_1-selective antagonist 1,3-dipropyl-8-cyclopentylxanthine (DPCPX; 1 μM). The inhibitory effects of CPA were unaltered in the added presence of the A_2-selective antagonist (E)-8-(3,4-dimethyloxystyryl)-1,3-dipropyl-7-methylxanthine (KF 17837; 1 μM). The A_{2A}-selective agonist 2-[p-(carboxyethyl)-phenethylamino]-5′-N-ethylcarboxamidoadenosine (CGS 21680), over the concentration range 1 nM to 10 μM, did not significantly alter ACh release when given alone or in the presence of DPCPX or KF 17837. These data suggest that the A_{2A} receptors previously identified in rat cortex are not functionally coupled to modulation of ACh release in this tissue. This does not exclude that these receptors may regulate the release of other neurotransmitters.

Adenosine is an endogenous neuromodulator (Dunwiddie, 1985; Fredholm and Hedqvist, 1980) which exerts its effects *via* a family of G-protein-coupled receptors of which four subtypes are currently recognized: A_1, A_{2A}, A_{2B} and A_3 (Fredholm *et al.*, 1994). The A_1 receptor is widely distributed in the brain, with high levels present in the cerebellum, hippocampus and some layers of the cortex (Fastbom *et al.*, 1987). A_{2A} receptors, by contrast, show a much more restricted pattern of distribution and were assumed to be confined to the dopamine-rich regions of the basal forebrain (Bruns *et al.*, 1986; Jarvis and Williams, 1989; Martinez-Mir *et al.*, 1991; Parkinson and Fredholm, 1990). Recent evidence indicates that their distribution may not be as limited as previously thought. Cunha *et al.* (1994b) demonstrated the presence of A_{2A} mRNA in the CA1, CA3 and dentate gyrus regions of the hippocampus and in the cerebral cortex. The presence of A_{2A} receptors in the hippocampus and cortex is also suggested by autoradiographic studies with the A_{2A}-selective agonist radioligand [^{3}H]CGS 21680 (Jarvis *et al.*, 1989; Hutchison *et al.*, 1989). Although most of these sites differ in several respects from classical A_{2A} receptors (Johansson and Fredholm, 1995; Johansson *et al.*, 1993), a minor component of the cortical [^{3}H]CGS 21680 binding could indeed be attributed to interactions with A_{2A} receptors, as suggested also by receptor binding studies (Cunha *et al.*, 1994a; Kirk and Richardson, 1995).

Adenosine A_1 receptors are known to mediate the inhibitory actions of adenosine on the release of many neurotransmitters in the central nervous system, including that of ACh, noradrenaline, dopamine and EAAs (see Fredholm and Dunwiddie, 1988). The role of adenosine A_2 receptors in the regulation of neurotransmitter release is more controversial. The A_{2A} agonist CGS 21680 was shown to the enhance ischemia-evoked release of the EAAs glutamate and aspartate from the cerebral cortex by use of the cortical cup technique (O'Regan *et al.*, 1992a), whereas the A_2 antagonist CGS 15943 inhibited the evoked release of these same neurotransmitters, possibly indicating a tonic influence of endogenous adenosine acting at excitatory A_{2A} receptors. Electrophysiological experiments indicate that CGS 21680 has a similar stimulatory effect on EAA release in the hippocampus (Sebastiao and Ribeiro, 1992), but see Lupica *et al.* (1990). In contrast to the effects on EAAs, CGS 21680 was shown to inhibit the release of GABA from the cortex (O'Regan *et al.*, 1992b) and from striatal synaptosomes (Kirk and Richard-

Received for publication June 6, 1995.
[1] This study was supported by Swedish Medical Research Council grant 2553.
[2] Recipient of a Canadian Medical Research Council postdoctoral fellowship.

ABBREVIATIONS: 2-CADO, 2-chloroadenosine; ACh, acetylcholine; CGS 21680, 2-[p-(carboxyethyl)-phenethylamino]-5′-N-ethylcarboxamidoadenosine; CGS 15943, 9-chloro-2-(2-furanyl)-5,6-dihydro-[1,2,4]-triazolo[1,5]quinazolin-5-imine monomethanesulfonate; CPA, N^6-cyclopentyladenosine; DPCPX, 1,3-dipropyl-8-cyclopentylxanthine; EAAs, excitatory amino acids; EGTA, ethylene glycol-bis(B-aminoethyl ether)-N,N,N′,N′-tetraacetic acid; GABA, γ-aminobutyric acid; KF 17837, (E)-8-(3,4-dimethyloxystyryl)-1,3-dipropyl-7-methylxanthine; TTX, tetrodotoxin.

son, 1994). Dopamine release from the rat striatum was inhibited by CGS 21680 (Lupica *et al.*, 1990), but only in high concentrations, and the effect was blocked by adenosine A_1 receptor antagonists (Jin *et al.*, 1993), which suggests that the effect was mediated through a locus other than the A_{2A} receptor.

The strongest evidence for a regulatory role of A_{2A} receptors comes from studies of ACh release. In synaptosome preparations from the striatum, ACh release was shown to be enhanced by agonists, including CGS 21680, that act at A_{2A} receptors (Brown *et al.*, 1990; Kirkpatrick and Richardson, 1993). However, in intact slices of rat striatum, CGS 21680 did not stimulate, but instead inhibited, the electrically evoked release of ACh (Jin *et al.*, 1993). CGS 21680, however, could reduce the inhibition of ACh release afforded by a dopamine D_2 agonist. Binding sites for CGS 21680 were found to copurify with cholinergic synaptosomes in one study (James and Richardson, 1993). On the other hand, several studies have failed to demonstrate the presence of A_{2A} receptor mRNA in striatal cholinergic neurons; instead the receptor mRNA is enriched in a subset of the medium-sized GABAergic neurons (Fink *et al.*, 1992; Johansson *et al.*, 1994; Schiffmann *et al.*, 1991). Release of acetylcholine from motor nerve endings and from hippocampus is stimulated by activation of A_{2A} receptors (Correia-de-Sá and Ribeiro, 1994; Cunha *et al.*, 1994b, c).

Because adenosine A_{2A} receptors have been identified in the rat cortex and because ACh release in other brain regions has been reported to be modulated by A_{2A} receptors, we have examined adenosine receptor-mediated regulation of ACh release from rat cortical slices.

Methods

Preparation of rat cortex slices. Male Sprague-Dawley rats (200–250 g) were housed in controlled conditions with 12-hr day-night cycles and food and water available *ad libitum*. Rats were killed by decapitation and the brain rapidly excised. After dissection of the cerebral cortex, 0.4-mm slices were made with a McIlwain tissue chopper operated manually. Slices were placed in 10 ml of Krebs' solution, washed twice for 30 min at 20°C and repeated once again at 37°C. The composition of the Krebs' solution was as follows (in mM): NaCl, 118; KCl, 4.85; $CaCl_2$, 1.3; KH_2PO_4, 1.15; $NaHCO_3$, 25; $MgSO_4$, 1.15 and glucose, 11.1. Krebs' solution was gassed continuously with a 95% O_2/5% CO_2 mixture to maintain pH. The slices were incubated at 37°C for 30 min with [^{14}C]choline (2 mCi/ml = 37 μM), loaded into chambers and superfused continuously with Krebs' solution at a flow rate of 0.5 ml/min. All solutions used from this point onward contained hemicholinium-3 (10 μM). After a 2-hr equilibration period, perfusate was sampled every 5 min (2.5 ml) and mixed with 8 ml of scintillation cocktail (Ready Safe, Beckman, Fullerton, CA). The radioactivity in each fraction was measured with a scintillation counter (Rackbeta, LKB Wallac, Turka, Finland). After each experiment, the cortical slices were removed from the chambers and homogenized by boiling for 2 min in NaOH (2 M). After neutralization with HCl (5 M) and buffering with Tris (1 M), these samples were also mixed with scintillation cocktail and counted to determine the total tissue content of [^{14}C]ACh in each slice.

Electrical stimulation of rat cortical slices. After a 15-min period (three samples) of basal release, ACh release was evoked by biphasic electrical stimulation (20 V, 2 msec duration) for 5 min. The slices were stimulated two times, separated by an unstimulated interval of 30 min. A stimulation frequency of 0.2 Hz was chosen.

Between stimulation periods S_1 and S_2, 30 min after sampling had begun, Krebs' buffer was changed to one containing the drug(s) to be examined. Experiments to determine the Ca^{++} sensitivity of ACh release used a Ca^{++}-free solution to which the calcium chelator EGTA (75 μM) was added. The involvement of Na^+ channels in evoked ACh release was investigated using TTX (10 μM). Carbachol (100 μM), alone or in the added presence of atropine (10 μM), was used to examine the influence of muscarinic receptors on ACh release.

Experiments designed to examine modulation of ACh release by adenosine receptor agonists used the A_1-selective agonist CPA, the A_{2A}-selective agonist CGS 21680 and the nonselective agonist 2-CADO over appropriate concentration ranges. Attempts to antagonize any observed effects of the agonists above involved perfusion with the agonist in the added presence of the A_1-selective antagonist DPCPX (1 μM) or the putative A_2-selective antagonist KF 17837 (1 μM). Control experiments were also performed to examine the actions of the antagonist in the absence of agonist.

Determination of [^{14}C]ACh in total ^{14}C release. In one series of experiments, the contribution of [^{14}C]ACh to the total amount of ^{14}C-efflux was determined. The methods used here are similar to those of Cunha *et al.* (1994d). Briefly, perfusate was collected as usual with the exception that neostigmine (20 μM) was present for the duration of the experiment. Samples (2.5 ml) were vacuum centrifuged overnight and reconstituted in 500 μl of distilled H_2O. A 200-μl aliquot was combined with 800 μl of phosphorylation buffer (10 mM sodium phosphate buffer, pH 7.9, 10 mM $MgCl_2$, 10 mM ATP, 0.01 U/ml choline kinase) and incubated at 20°C for 30 min. To stop the reaction, 4 ml of ice-cold phosphate-buffered saline (130 mM NaCl and 3 mM KH_2PO_4) was added followed by further addition of 2 ml of tetraphenylboron (also known as Kalignost; 5g/l in acetonitrile). This was shaken for approximately 1 min and the resulting organic fraction was combined with 8 ml of OptiScint Hi Safe (LKB Wallac) and counted for ^{14}C-radioactivity. Only [^{14}C]ACh is extracted into the organic phase under these conditions and the contribution of [^{14}C]ACh to total measured ^{14}C could be subsequently calculated.

Data analysis. Total evoked release of [^{14}C]ACh was calculated as the sum of evoked release above basal release. Total evoked release was calculated for each period of stimulation (S_1 and S_2). Because all drugs were added after S_1, changes in the S_2/S_1 ratio were used to measure the effect of a compound on ACh release. Experiments investigating the actions of a drug were always paired with control stimulation (no drugs present).

All data were the result of experiments that used six to eight slices from at least three animals. During experiments in which the effect of one drug was compared with control conditions, an unpaired, two-tailed Student's t test was used. When the effect of more than one drug was being examined and compared with control, ANOVA followed by a Bonferroni post test for multiple comparisons was used. Statistical significance was accepted at $P < .05$.

Chemicals. With the exception of the following, all chemicals used were purchased from BDH (Poole, England) or Merck (Darmstadt, Germany) and were of the highest grade possible. ATP, atropine, carbachol, choline kinase, EGTA, hemicholinium-3, neostigmine bromide, sodium tetraphenyborate and TTX were obtained from Sigma Chemical Co. (St. Louis, MO). 2-CADO, CGS 21680, CPA and DPCPX were obtained from Research Biochemicals International (Natick, MA). KF 17837 was the kind gift of Dr. Fumio Suzuki (Kyowa Hakko Kogyo, Japan).

CPA and DPCPX were dissolved in ethanol, CGS 21680 was dissolved in 50% aqueous DMSO and KF 17837 was dissolved in DMSO. All other chemicals were dissolved in distilled water. A dilution factor of at least 1000 was used between stock solutions and the final concentration of drug in Krebs' solution. There was no effect of the solvents at this dilution.

Results

Electrically stimulated ACh release from rat cortex slices. Separation of [^{14}C]ACh from [^{14}C]choline indicated that, during basal release, the contribution of [^{14}C]ACh to total measured ^{14}C was 58.1 ± 8.4% (mean ± S.E.M.). During stimulated release, this value increased to 86.7 ± 2.6% for S_1 and 76.6 ± 1.4% for S_2. Because evoked release was about twice that of basal (fig. 1), these results provide evidence that, under these conditions, electrically evoked ^{14}C-efflux is essentially a measure of ACh release. Accordingly, the two terms are used interchangeably from this point onward (see also Richardson and Szerb, 1974).

As shown in figure 1, the electrically stimulated release of ACh from superfused rat cortical slices was both repeatable and reversible. Basal release values returned to normal after each stimulation period, with the S_2/S_1 for this particular experiment essentially 100% (99.3 ± 5.8%). The average S_2/S_1 for 21 series of experiments was 86.8 ± 1.6%. Figure 1 also demonstrates that electrically stimulated release during S_2 was not seen in the absence of extracellular Ca^{++} (and the added presence of 75 μM EGTA), with evoked release under these conditions being 9.7 ± 2.7% of control. This release was significantly different ($P < .05$) from control.

Electrically stimulated release of ACh was also sensitive to the presence of the neurotoxin, TTX (10 μM). Release from slices superfused with TTX was significantly ($P < .05$) reduced to 23.0 ± 5.3% of control. Perfusion of cortical slices with carbachol (100 μM) inhibited ACh release to 2.0 ± 1.5% of control. Further perfusion in the added presence of atropine (10 μM) reversed this inhibition. Atropine alone did not stimulate electrically evoked release of ACh (not shown).

Adenosine receptor-mediated modulation of ACh release. Perfusion with the A_1-selective adenosine receptor agonist, CPA, over the concentration range of 0.1 nM to 100 μM led to a dose-dependent inhibition of ACh release from electrically stimulated rat cortical slices. The EC_{50} for CPA was 27 nM with maximal inhibition (60%) occurring at 10 μM. A 10-fold increase in concentration of CPA to 100 μM did not result in a further increase of inhibition. CPA-mediated inhibition was significant ($P < .05$) at concentrations of 200 nM or higher. Further addition of the A_1-selective antagonist DPCPX (1 μM) caused a rightward shift of the dose-response curve to CPA (fig. 2A). This attenuation of CPA-mediated inhibition was significant ($P < .05$) at lower concentrations of CPA, but not at the higher concentration of the agonist. The EC_{50} for CPA in the added presence of DPCPX was 5.0 μM. DPCPX (1 μM) alone did not lead to a significant change in ACh release (93.3 ± 12.3%). A lower concentration of DPCPX (100 nM) did not significantly antagonize the effect of 200 nM or 1 μM CPA on ACh release (fig. 2A).

Perfusion in the combined presence of CPA (1 μM) and the A_{2A}-selective antagonist KF 17837 (1 μM) did not affect CPA-mediated inhibition of ACh release (fig. 2A). Release in the presence of KF 17837 (1 μM) alone was not significantly different (104.4 ± 6.5%) from control.

Perfusion with Krebs' solution containing CGS 21680 (1 nM to 10 μM) did not lead to any significant change in ACh release. This lack of effect of CGS 21680 was not influenced by further addition of the A_1-selective antagonist DPCPX (1 μM) for all concentrations of the agonist (fig. 2B). Similarly, release in the presence of 1 μM CGS 21680 was unaltered in the added presence of KF 17837 (fig. 2B).

The nonselective adenosine receptor agonist 2-CADO at a concentration of 100 nM caused a slight inhibition of ACh release, whereas 1 μM 2-CADO led to a significant ($P < .05$)

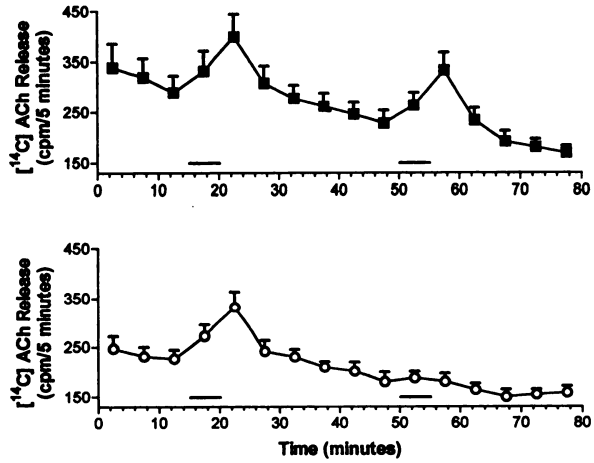

Fig. 1. The time profile for release of [^{14}C]ACh from electrically stimulated slices of rat cortex. A 5-min stimulation of biphasic pulses (2 mA, 20 V) with a frequency of 0.2 Hz was given at the horizontal bars. The top figure shows release during control perfusion; the bottom figure shows release during perfusion where normal Krebs' solution was replaced with Ca^{++}-free Krebs' (75 μM EGTA added) during S_2. Data represent the average of eight slices. Error bars are ± S.E.M.

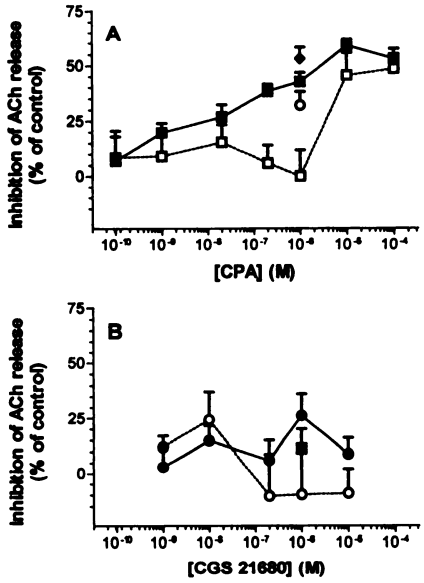

Fig. 2. (A) Concentration-dependent inhibition of [^{14}C]ACh release by the A_1-selective adenosine receptor agonist CPA. Closed squares show inhibition in the presence of CPA alone; open squares show inhibition in the added presence of the A_1-selective antagonist DPCPX (1 μM); the open circle shows inhibition with CPA and DPCPX (100 nM) and the closed diamond shows inhibition with CPA and KF 17837 (1 μM). Data represent the average of eight slices. Error bars are ± S.E.M. (B) Lack of inhibition of ACh release by the A_{2A}-selective adenosine receptor agonist CGS 21680. Closed circles show inhibition in the presence of CGS 21680 alone; open circles show inhibition in the added presence of the A_1-selective antagonist DPCPX and the closed square shows inhibition in the added presence of KF 17837 (1 μM). Data represent the average of eight slices. Error bars are ± S.E.M.

inhibition of ACh release. The inhibitory effects of 1 μM 2-CADO were attenuated upon further addition of DPCPX, although this effect did not achieve significance. Co-perfusion of 2-CADO and KF 17837 caused a significant reversal of inhibition compared with perfusion in the presence of 2-CADO alone (fig. 3).

Discussion

Electrical stimulation with the parameters used in this study caused a reproducible and calcium-dependent release of ACh from rat cortical slices which was clearly enhanced above base-line release values. Evoked release was reduced by the Na^+ channel blocker, TTX (10 μM). Because the release of neurotransmitters resulting from direct stimulation of nerve endings is known to be insensitive to TTX, these data may suggest that only a small component of the ACh release measured in these experiments is due to direct stimulation of nerve endings. Evoked ACh release could be blocked by the addition of the cholinergic agonist carbachol (100 μM), whereas addition of the muscarinic antagonist atropine at a concentration that blocked the effect of carbachol (10 μM) had no effect *per se*. Thus, there was no evidence for an important muscarinic autoreceptor regulation of evoked acetylcholine release under the present conditions, although an inhibitory influence of muscarinic receptor activation was demonstrated.

Perfusion in the presence of the A_1-selective adenosine agonist CPA led to the expected inhibition of evoked ACh release. This inhibition was dose dependent, with a calculated EC_{50} of 27 nM. A rightward shift of the dose-response curve to CPA was seen in the added presence of the A_1-selective antagonist DPCPX at a concentration of 1 μM. This provides further evidence that the adenosine receptor-mediated inhibition of ACh release is due to selective interactions with A_1 adenosine receptors. Because the ability of DPCPX to attenuate CPA-mediated responses could be overcome by increasing concentrations of agonist, the interaction between the two compounds appears to be competitive. The observation that DPCPX alone had no effect provides evidence that, under the conditions used in this study, endogenous levels of adenosine are not sufficient to cause significant activation of presynaptic inhibitory adenosine A_1 receptors. This provides indirect evidence that, under these experimental conditions,

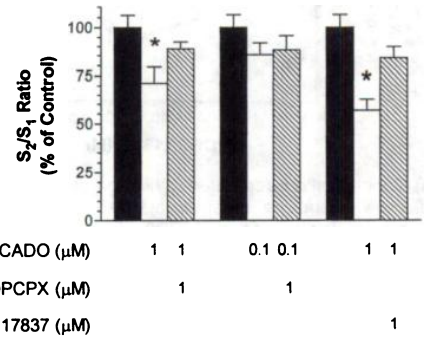

Fig. 3. A bar graph demonstrating inhibition of ACh release by the nonselective adenosine receptor agonist 2-CADO, as well as the effects of further addition of the A_1-selective antagonist DPCPX or the A_2-selective antagonist KF 17837. Data are from six to eight experiments. Error bars represent ± S.E.M.

neuromediators released from nerve endings are sufficiently distant from their site of action that they cannot exert an influence on the release of ACh.

In contrast to CPA, the A_{2A}-selective agonist CGS 21680 did not cause significant modulation of ACh release, because neither significant inhibitory nor stimulatory effects were observed. At the highest concentrations used (1–10 μM) there was a tendency toward an inhibition of evoked ACh release, although this effect did not reach significance. This is in agreement with previous reports that CGS 21680, at high concentrations, seems to act as an agonist also at A_1 receptors (*e.g.*, Jin *et al.*, 1993; Lupica *et al.*, 1990). Indeed, inhibition tended to be lower in the presence of the A_1 antagonist DPCPX than in its absence, although the differences did not reach significance.

CGS 21680 was tested over the concentration range of 1 nM to 10 μM, allowing for potential effects at both high- and low-affinity A_2 receptors, as well as nonspecific effects at A_1 receptors. Kirkpatrick and Richardson (1993) demonstrated that the ability of CGS 21680 to enhance the release of acetylcholine from rat striatal synaptosomes was biphasic, with effects of the agonist diminishing at concentrations over 100 nM. These authors also demonstrated maximal A_{2A}-mediated effects at 1 and 10 nM. We have used these same concentrations and found no effects of the agonist on ACh release in the cortex. Similarly, the nonselective agonist, 2-CADO, did not stimulate ACh release during simultaneous blockade of A_1 receptors with DPCPX, even though an inhibitory effect of the agonist could be demonstrated in the absence of antagonist. These experiments indicate a lack of inhibitory or excitatory influence by A_{2A} receptors on the release of ACh.

In an earlier study, Pedata *et al.* (1986) concluded that A_1 receptors mediated the inhibition of ACh release from cortical synaptosomes. Interestingly, under conditions in which the A_1 agonist CHA inhibited ACh release by almost 50%, NECA at concentrations up to 100-fold greater than that of CHA was ineffective. Because NECA is only about 10 times less potent than CHA at A_1 receptors, but differs from CHA in that it also activates A_2 receptors, the results of Pedata *et al.* (1986) suggest the possibility that the actions of inhibitory A_1 receptors might be counteracted by stimulatory A_2 receptors. Although this may be true for cortical synaptosomes stimulated by potassium, the present study, with the A_{2A}-selective agonist CGS 21680 and antagonist KF 17837, shows that similar effects are not seen with electrically stimulated nerve endings in a cortical slice preparation.

The likely explanation for the absence of effect of CGS 21680 on ACh release is that adenosine A_{2A} receptors in the cortex and the binding sites for CGS 21680 are not functionally coupled to the release of ACh. This agrees with the finding that CGS 21680 does not affect basal or potassium-evoked ACh release into cortical cups (Phillis *et al.*, 1993a) or release evoked by ischemia (Phillis *et al.*, 1993b). This does not exclude that these receptors regulate the release of other neurotransmitters, possibly EAAs. O'Regan *et al.* (1992a) have demonstrated an enhancement of glutamate and aspartate release from cortical neurons in the presence of CGS 21680. In addition, Simpson *et al.* (1992) provided evidence that the release of these same neurotransmitters was inhibited by the A_2 antagonist CGS 15943 in the absence of adenosine agonist, not only supporting the data of O'Regan *et al.*,

but also suggesting a tonic excitatory influence of adenosine on the release of EAAs. Such A_{2A}-mediated actions on the release of EAAs, combined with the lack of effect of A_{2A} receptors on the release of ACh in the current study, demonstrate a selectivity of action of A_{2A} agonists in the cortex. This suggests that the A_{2A} receptor binding previously demonstrated in the cortex (Johansson *et al.*, 1993) either occurs only to EAA neurons, or if the demonstrated binding is occurring at a site on cholinergic neurons, it is not functionally coupled to the release of ACh.

These data provide further evidence for the existence of A_1 adenosine receptors on the cholinergic neurons of the rat cortex. These receptors are functionally coupled to the inhibition of ACh release. No evidence for a role of A_{2A} receptors in the regulation of cortical ACh release was obtained, despite strong evidence that these receptors may regulate ACh release in other brain regions. Thus, there may be clear differences in adenosine receptor-mediated regulation of transmitter release between different nerve populations in the central nervous system with the same principal neurotransmitter. It can also be concluded that if the A_{2A} receptors previously identified in the cortex are involved in regulating neurotransmitter release, the affected transmitter is not ACh.

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