Activation of Hippocampal Adenosine A_3 Receptors Produces a Desensitization of A_1 Receptor-Mediated Responses in Rat Hippocampus

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The adenosine A_3 receptor is expressed in brain, but the consequences of activation of this receptor on electrophysiological activity are unknown. We have characterized the actions of a selective adenosine A_3 receptor agonist, 2-chloro-N^6-(3-iodobenzyl)-adenosine-5′-*N*-methyluronamide (Cl-IB-MECA), and a selective A_3 receptor antagonist, 3-ethyl-5-benzyl-2-methyl-4-phenylethynyl-6-phenyl-1,4-(±)-dihydropyridine-3,5-dicarboxylate (MRS 1191), in brain slices from rat hippocampus. In the CA1 region, activation of A_3 receptors had no direct effects on synaptically evoked excitatory responses, long-term potentiation, or synaptic facilitation. However, activation of A_3 receptors with Cl-IB-MECA antagonized the adenosine A_1 receptor-mediated inhibition of excitatory neurotransmission. The effects of Cl-IB-MECA were blocked by pretreatment with MRS 1191, which by itself had no effect on A_1 receptor-mediated responses. The presynaptic inhibitory effects of baclofen and carbachol, mediated via $GABA_B$ and muscarinic receptors, respectively, were unaffected by Cl-IB-MECA. The maximal response to adenosine was unchanged, suggesting that the primary effect of Cl-IB-MECA was to reduce the affinity of adenosine for the receptor rather than to uncouple it. Similar effects could be demonstrated after brief superfusion with high concentrations of adenosine itself. Under normal conditions, endogenous adenosine in brain is unlikely to affect the sensitivity of A_1 receptors via this mechanism. However, when brain concentrations of adenosine are elevated (e.g., during hypoxia, ischemia, or seizures), activation of A_3 receptors and subsequent heterologous desensitization of A_1 receptors could occur, which might limit the cerebroprotective effects of adenosine under these conditions.

Key words: adenosine; A_3 receptor; A_1 receptor; protein kinase C; hippocampus; electrophysiology; receptor desensitization

The adenosine A_3 receptor was originally identified based on cloning experiments using degenerate oligonucleotide probes. A previously unknown receptor of the G-protein-coupled family that showed significant overall homology to the adenosine A_1 and A_{2a} receptors (Meyerhof et al., 1991) was identified pharmacologically as an adenosine receptor (Zhou et al., 1992). Various species homologs of this receptor have been cloned, including the human A_3 receptor (Salvatore et al., 1993). The A_3 receptors from different species show different pharmacological properties, the most noteworthy being the rat A_3 receptor, which has a very low affinity for xanthine-based adenosine receptor antagonists such as theophylline. In addition, many adenosine agonists have A_3 receptor affinities that are typically much lower than their corresponding affinities at the adenosine A_1 receptor (Zhou et al., 1992) but not at the adenosine A_{2a} receptor (van Galen et al., 1994). Although A_3 receptors can inhibit adenylyl cyclase when expressed in CHO cells (Zhou et al., 1992); this effect does not appear to be very robust with the native receptor (Abbracchio et al., 1995). Instead, A_3 receptor activation has been linked to the activation of phospholipase C and elevation in inositol phosphate levels (Ali et al., 1990; Ramkumar et al., 1994), and this is the case in brain as well (Abbracchio et al., 1995). Activation of the A_3 receptor would therefore be expected to lead to the activation of protein kinase C (PKC) via this type of mechanism.

Although the A_3 receptor is expressed in brain in significant amounts (Zhou et al., 1992; De et al., 1993), its physiological effects in the CNS at the cellular level are unknown. The recent development of agonists that are highly selective for the A_3 receptor, such as 2-chloro-N^6-(3-iodobenzyl)-adenosine-5′-*N*-methyluronamide Cl-IB-MECA, which is ~2500-fold selective for the A_3 versus the A_1 receptor, and 1400-fold selective for the A_3 versus the A_{2a} receptor (Jacobson et al., 1995), has made it possible to investigate the effects of activation of this receptor in brain. Behavioral studies have demonstrated that A_3-selective agonists depress locomotor activity (Jacobson et al., 1993), but effects of A_3 receptor activation on neuronal activity at the cellular level have not been described.

The A_3 receptor is expressed at relatively low levels in brain, but there are significant brain regional differences in the levels of A_3 receptor mRNA. In the rat, the hippocampus and cerebellum show the highest levels of A_3 mRNA in brain (De et al., 1993). The absolute level of A_3 receptor expression shows considerable species variation but is generally less than that of other adenosine receptors. The level of A_3 receptor binding in mouse hippocampus is quite high (220 fmol/mg protein) (Jacobson et al., 1993) but is still well below that of the A_1 receptor (1100 fmol/mg protein) (Lee et al., 1983; Cunha et al., 1995) or the A_{2a} receptor (350

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fmol/mg protein) (Cunha et al., 1996; Johansson and Fredholm, 1995). Because of the relatively high levels of expression of A_3 receptors in hippocampus and because the responses to A_1 and A_2 receptor activation have been well characterized in this brain region (Dunwiddie, 1985; Greene and Haas, 1991), we have investigated the electrophysiological actions of Cl-IB-MECA in this brain region.

MATERIALS AND METHODS

Slice preparation. Hippocampal slices were obtained from 6- to 8-week-old, male Sprague Dawley rats (Sasco Animal Laboratories, Omaha, NE) using standard techniques (Dunwiddie and Lynch, 1978; Dunwiddie and Hoffer, 1980). Animals were decapitated, and the hippocampus was dissected free from the whole brain, and 400 μm slices were cut from the middle third of the hippocampus with a TC-2 tissue chopper (Sorvall). Slices were initially transferred to an interface holding chamber maintained at 33°C to equilibrate. At least 1 hr after preparation, the slices were transferred to a submersion recording chamber (1 ml volume), where they were placed on a nylon net and superfused (2 ml/min) with medium containing (in mM): 124 NaCl, 3.3 KCl, 1.2 KH_2PO_4, 2.4 $MgSO_4$, 2.5 $CaCl_2$, 10 D-glucose, and 25.7 $NaHCO_3$, pH 7.4. The perfusion medium was gassed with humidified 95% O_2/5% CO_2 and maintained at a temperature of 33–34°C.

Electrophysiological recordings. Extracellular electrophysiological recordings of the field excitatory postsynaptic potentials (fEPSP) and population spikes (PS) were made using glass microelectrodes (2–4 MΩ) filled with 3 M NaCl and placed in either stratum radiatum or stratum pyramidale of the CA1 region. Twisted bipolar nichrome wire stimulating electrodes were placed in stratum radiatum near the border of the CA1 and CA2 regions. Stimuli consisting of 0.2 msec square wave pulses were delivered to the synaptic pathway at 15 sec intervals. The stimulation voltage was adjusted individually for each slice to produce fEPSP and PS that were ~1-2 mV in amplitude, which were ~20% of the maximal responses that could be evoked. To test paired-pulse facilitation, the Schaffer collateral and commissural afferents were stimulated with pairs of pulses every 15 sec, and the interpulse intervals were 60 msec. Long-term potentiation (LTP) was induced with high-frequency stimulation(100 Hz train/1 sec). All electrodes were positioned visually. Responses were recorded using an AC amplifier, and a computer was used to digitize and store the responses for further analysis.

At least 10–15 min of stable baseline responses was obtained in each experiment before drug applications began. Drugs were made up at 100–2000 times the desired final concentration and added directly to the flow of the superfusion medium with a calibrated syringe pump to achieve the desired final concentration. The superfusion rate (2 ml/min) was monitored with a glass flowmeter (Cole-Parmer) during each experiment, and the flowmeter was calibrated periodically to ensure that the final concentrations of drugs in the superfusate were accurate. Cl-IB-MECA, MRS1191, and 8-(3-chlorostyryl)caffeine (CSC) were dissolved initially in 100% DMSO and diluted such that the final concentration of DMSO in the bath was 0.05%. The other drugs were made up in distilled water. In a few experiments, a high (100 μM) concentration of Cl-IB-MECA was tested; because of limited availability of drug, these experiments were conducted in nonsuperfused slices. In these experiments, Cl-IB-MECA was added directly to a nonsuperfused slice chamber, and 40 min later the slices were tested with the addition of different concentrations of adenosine. Because of the limited solubility of Cl-IB-MECA, there was a some precipitation of drug at the nominal 100 μM concentration; this was not apparent at 10 μM, thus the final concentration of Cl-IB-MECA in these experiments was between 10 and 100 μM.

The peak fEPSP and PS amplitudes were determined for individual responses and then averaged during the predrug control, during drug superfusion, and during the postdrug washout period; at least 10 responses were included in each average. In all of the experiments, the data were analyzed as mean percentage change in response amplitude when compared with responses obtained during the control period. Effects of drugs were analyzed between groups, using the unpaired Student's *t* test and nonparametric test (Mann–Whitney test).

Chemicals. Adenosine was obtained from Sigma (St. Louis, MO); CSC, baclofen, CGS21680, and 5′-*N*-ethyl-carboxamidoadenosine (NECA) were obtained from Research Biochemicals (Natick, MA); carbachol was obtained from ICN K & K Laboratories; and Cl-IB-MECA and 3-ethyl-5-benzyl-2-methyl-4-phenylethynyl-6-phenyl-1,4-(±)-dihydropyridine-3,5-dicarboxylate (MRS 1191) were synthesized as described (Jacobson et al., 1995; Jiang et al., 1996).

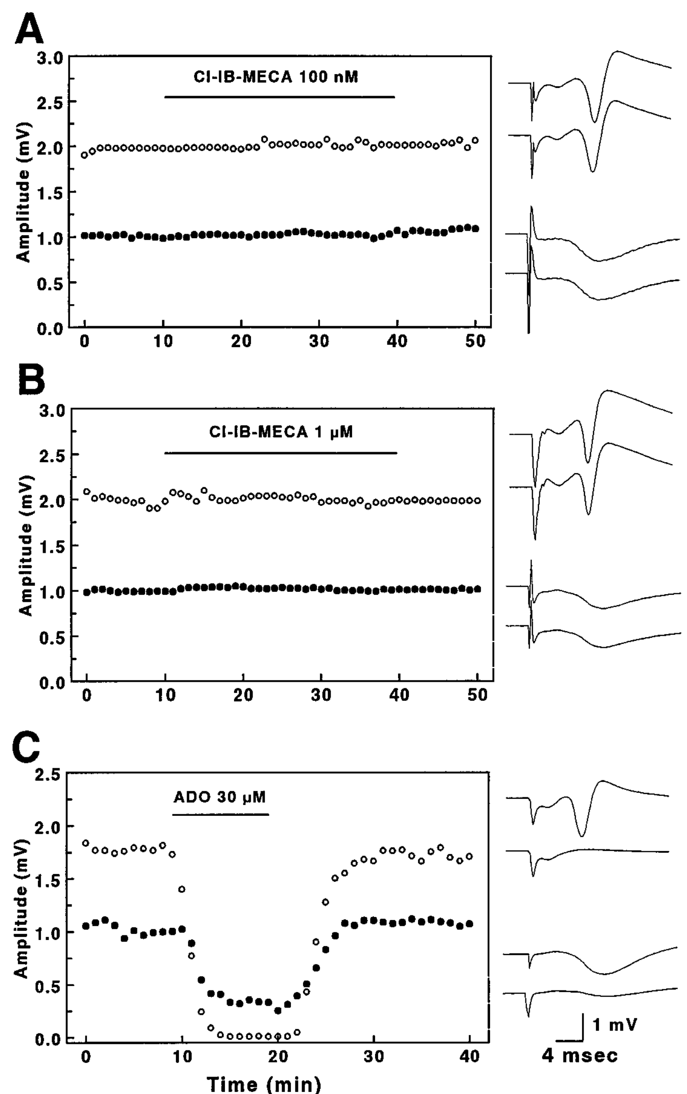

Figure 1. Effects of Cl-IB-MECA on hippocampal synaptic physiology. Slices were superfused with 100 nM (*A*) or 1 μM (*B*) Cl-IB-MECA, and the effects on PS (*open circles*) or fEPSP (*filled circles*) responses were determined. Results from individual slices are illustrated. Neither fEPSP nor PS responses were affected by treatment with either concentration of Cl-IB-MECA. On the other hand, superfusion with 30 μM adenosine (*C*) completely inhibited the PS and inhibited the fEPSP component of the response by ~70%. On the *right* are signal averaged responses obtained before and during superfusion with Cl-IB-MECA (*A*, *B*) or adenosine (*ADO*, *C*). The top response of each pair is the control, the bottom in the presence of drug. PS responses recorded from the cell layer are shown *above*, and fEPSP responses from stratum radiatum are shown *below*. Adenosine eliminated the negative going PS response (*C*, *top*) and reduced the fEPSP (*C*, *bottom*).

RESULTS

In initial studies, we characterized the direct actions of an adenosine A_3 receptor-selective agonist, Cl-IB-MECA, on electrophysiological activity in the CA1 region of rat hippocampal slices. Superfusion of Cl-IB-MECA at concentrations of up to 1 μM for periods as long as 30 min had no apparent effect on synaptically evoked responses in this brain region (Fig. 1). The net effect of 100 nM Cl-IB-MECA was a 3.8 ± 1.3% decrease in the fEPSP

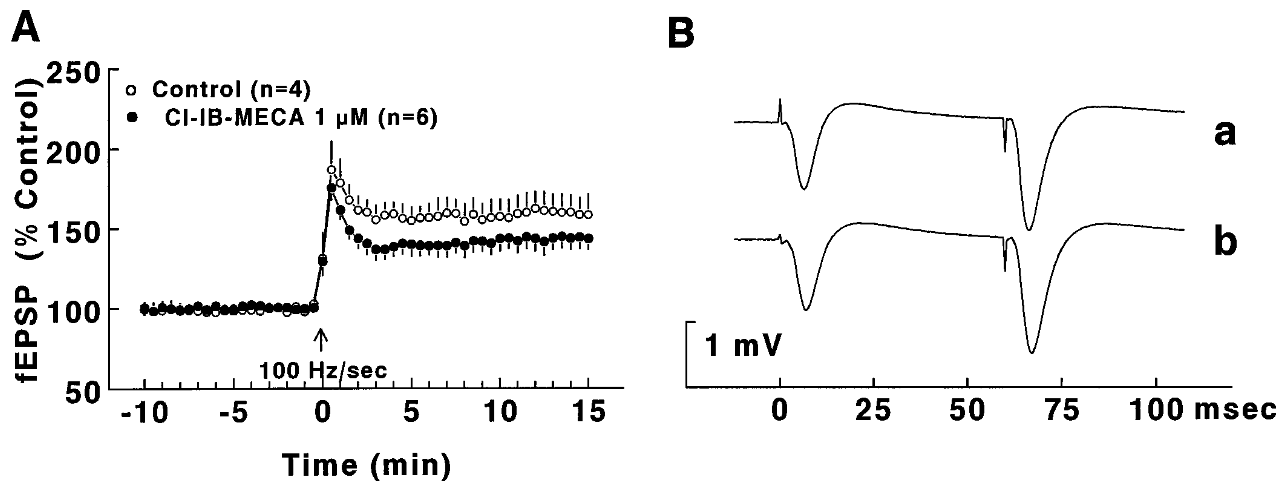

Figure 2. Effects of Cl-IB-MECA on hippocampal synaptic plasticity. In *A*, slices were stimulated with a train of 100 Hz stimulation for 1 sec to induce LTP of the Schaffer collateral and commissural synapses. Under control conditions, this induced a reliable and persistent enhancement of the fEPSP amplitude. Pretreatment with 1 μM Cl-IB-MECA for 30 min before the stimulation train had no significant effect on the amplitude of the ensuing LTP. The ensemble averages for all the slices tested in this manner are illustrated in *A*. *B* illustrates hippocampal paired-pulse facilitation; when excitatory inputs to the CA1 region are stimulated twice in rapid succession, there is a significant potentiation of the second synaptic response called paired-pulse facilitation (Creager et al., 1980). Responses are illustrated from a control slice (*B*, *a*) and from a slice incubated in 1 μM Cl-IB-MECA (*B*, *b*) and tested with a 60 msec interpulse interval. The degree of facilitation (65 and 63%, respectively, in the examples shown) was not significantly different in the two conditions.

amplitude in a group of 17 slices treated with the protocol illustrated in Figure 1 ($p > 0.1$), whereas 1 μM Cl-IB-MECA produced a 2.3 ± 1.8% decrease in the fEPSP response ($n = 33$; $p > 0.1$). This is in contrast to adenosine, which inhibits both PS and fEPSP responses via actions mediated by presynaptic adenosine A_1 receptors (Dunwiddie and Hoffer, 1980). The lack of effect of Cl-IB-MECA on these responses suggested that it has no significant actions on A_1 receptors at concentrations of up to 1 μM. LTP is a persistent form of synaptic plasticity that can be induced by high-frequency stimulation of the Schaffer collateral and commissural afferents to the CA1 region. When slices were pretreated with Cl-IB-MECA and then stimulated with a 100 Hz/1 sec stimulation train, there was no significant effect on the magnitude or persistence of the enhancement of the fEPSP response after such stimulation (Fig. 2*A*). Another form of short-term synaptic plasticity that occurs at this synapse is paired-pulse facilitation, which is an enhancement of a second synaptic response that occurs when the Schaffer collateral and commissural afferents are stimulated twice in rapid succession and which is thought to reflect the persistence of Ca^{2+} in the nerve terminal after the initial stimulus. The magnitude of paired-pulse facilitation with a 60 msec paired-pulse interval in control slices (52 ± 4%, $n = 11$) was not significantly different from that in slices superfused with 1 μM Cl-IB-MECA (55 ± 3%, $n = 11$, $p > 0.1$) (Fig. 2*B*).

Because of the lack of direct responses to Cl-IB-MECA, we determined whether it was able to modify responses mediated via A_1 receptors in hippocampus. Adenosine normally acts on A_1 receptors to inhibit synaptically evoked excitatory responses in the CA1 region (Fig. 1*C*) (Reddington et al., 1982; Dunwiddie and Fredholm, 1989). Because such responses typically show no desensitization and are highly repeatable (Figs. 3*A*, 4*A*), this system was used to test for interactions between A_1 and A_3 receptors. Slices were superfused initially with 30 μM adenosine, a concentration that elicits an ~50% inhibition of the fEPSP response, and were then superfused with Cl-IB-MECA and tested again with 30 μM adenosine. Superfusion with both 100 nM and 1 μM Cl-IB-MECA significantly inhibited the fEPSP response to adenosine, with the 1 μM concentration almost completely blocking the effect of adenosine (Fig. 3*C*). This response depended on the order in which the drugs were tested; if fEPSPs were first inhibited with adenosine, and then Cl-IB-MECA was added, it did not antagonize the already established inhibitory response to adenosine.

To demonstrate that this effect was mediated via A_3 receptors, we examined the effects of the selective A_3 receptor antagonist MRS 1191 (Jiang et al., 1996). Slices were initially tested with adenosine alone (30 μM), then superfused with 10 μM MRS 1191 and retested with adenosine, and then superfused with MRS 1191 and Cl-IB-MECA and tested for a third time with adenosine, using the protocol illustrated in Figure 4*A*. Control slices were treated similarly, but the MRS 1191 was omitted. The A_3 antagonist MRS 1191 had no effect on the normal inhibitory response to adenosine (confirming that this concentration of MRS 1191 did not interact significantly with A_1 receptors), but it completely blocked the ability of Cl-IB-MECA to antagonize the adenosine response (Fig. 4*B*).

Because adenosine can be taken up and metabolized by neurons and glial cells, it was possible that Cl-IB-MECA disrupted the response to adenosine by increasing the rate of inactivation (and hence reducing the extracellular concentration) of adenosine in the brain slice. To determine whether this were the case, similar experiments were conducted using 20 nM NECA, a metabolically stable analog of adenosine that is not a substrate for the nucleoside transporter, but which is a potent agonist at the A_1 receptors that mediate the inhibitory effects of adenosine on synaptic transmission. As with adenosine, the response to NECA was significantly reduced by previous superfusion with Cl-IB-MECA, and the extent of the inhibition of adenosine and NECA responses of comparable magnitude was not significantly different (Fig. 5).

Adenosine is one of a number of presynaptic modulators that can inhibit synaptic transmission at Schaffer collateral and commissural synapses to the CA1 region. Acetylcholine acting via a muscarinic receptor and GABA acting via a $GABA_B$ receptor can also inhibit transmission at these synapses. To determine whether A_3 receptor activation selectively disrupted the presynaptic mod-

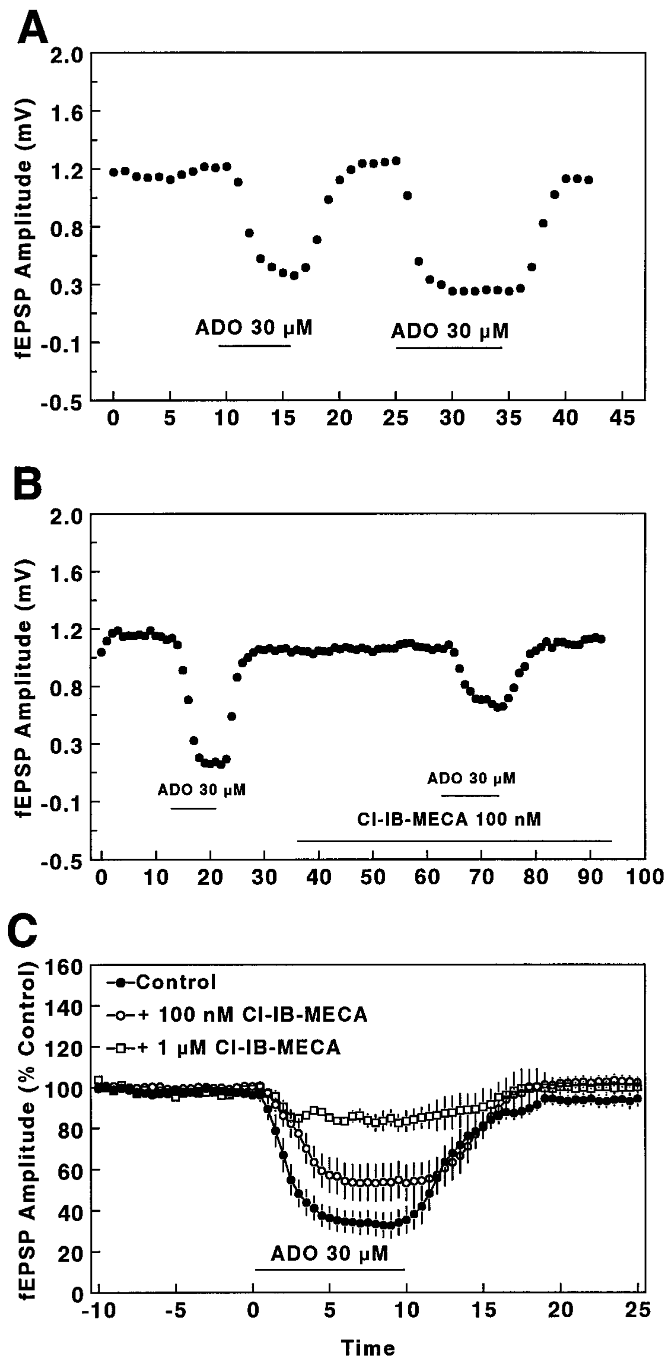

Figure 3. Cl-IB-MECA antagonizes the effects of adenosine on fEPSP responses. When slices were superfused repeatedly with 30 μM adenosine (*A*), the response to the second treatment with adenosine was an inhibition of the fEPSP amplitude comparable in magnitude to the first (i.e., there was no desensitization of the A_1 receptor-mediated inhibition). However, when the slice was pretreated with either 100 nM or 1 μM Cl-IB-MECA before the second adenosine superfusion, the response was markedly inhibited (*B*). Similar effects were observed when the order was reversed, i.e., when the initial test was with Cl-IB-MECA + adenosine, and then the adenosine was tested alone after washout of Cl-IB-MECA (data not shown). Ensemble averages are shown in *C* for all three conditions. The inhibition of the adenosine response by 100 nM Cl-IB-MECA was statistically significant ($p < 0.05$), as was the inhibition by 1 μM Cl-IB-MECA ($p < 0.0001$).

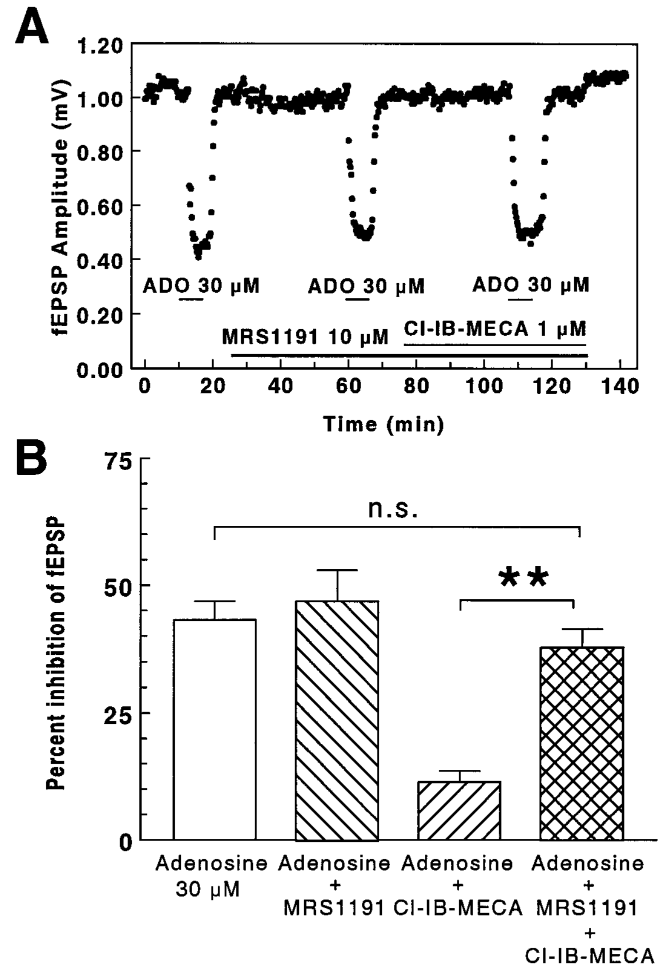

Figure 4. MRS 1191 selectively blocks A_3 receptor mediated responses. *A*, Slices were superfused with adenosine, MRS 1191, and Cl-IB-MECA as denoted by the *bars* at the *bottom* of the figure, while the fEPSP response was tested at 15 sec intervals. Superfusion with 10 μM MRS 1191 had no effect on the adenosine response, but it blocked the ability of Cl-IB-MECA to disrupt adenosine responses (compare Fig. 3*C*). *B*, Summary of experiments with MRS 1191 and Cl-IB-MECA. Each bar represents the percent inhibition of the fEPSP response by 30 μM adenosine in the presence of the indicated drugs; slices were tested with the protocol illustrated in *A* or with a similar protocol but without MRS 1191. Each bar is the mean ± SEM for eight slices tested with an identical protocol. *n.s.*, Not statistically significant, $^{**}p < 0.0001$.

ulatory effects of adenosine at these synapses, the ability of Cl-IB-MECA to block modulation by baclofen and carbachol was examined. Inhibitory responses to both of these agents were unaffected by Cl-IB-MECA pretreatment (Fig. 5), indicating that the effects of A_3 receptor activation were confined to modulatory effects mediated by the adenosine A_1 receptor.

Although Cl-IB-MECA is highly selective for A_3 receptors in ligand binding studies, one possibility was that when used in relatively high concentrations, Cl-IB-MECA was acting on adenosine receptors other than the A_3 subtype to disrupt the response to adenosine. The possibility that Cl-IB-MECA was an agonist at A_1 receptors was ruled out by the fact that it had no direct effect on fEPSP responses (Figs. 1, 6) and by its relatively low affinity for A_1 receptors as determined in ligand-binding experiments (Jacobson et al., 1995). Two different kinds of experiments were conducted to rule out mediation via A_{2a} receptors. First, pretreatment of slices with the selective A_{2a} receptor agonist CGS 21680

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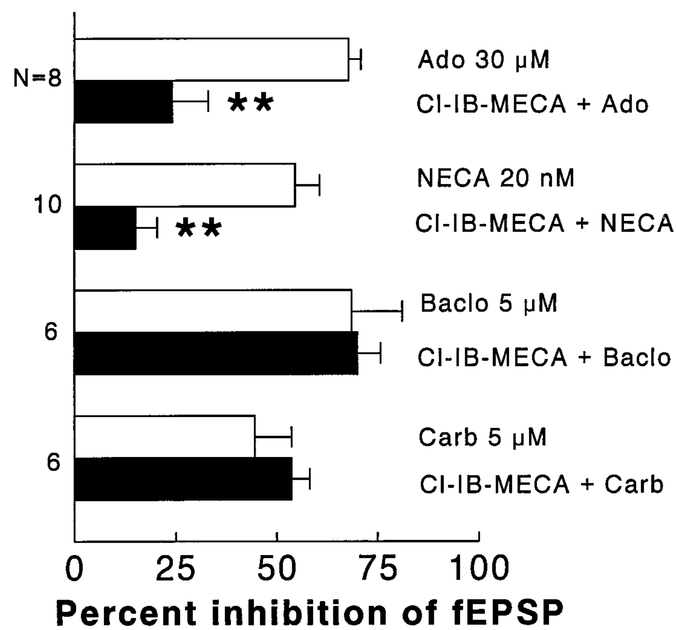

Figure 5. Cl-IB-MECA selectively disrupts the presynaptic modulatory effects of adenosine receptor agonists. Superfusion of slices with 30 μM adenosine, 20 nM NECA, 5 μM baclofen, or 5 μM carbachol (*open bars*) significantly inhibited the fEPSP response. Each bar shows the mean ± SEM inhibition of the response with each of the indicated drugs (the number of slices tested is shown to the *left* of each pair of *bars*). *Solid bars* indicate slices that were pretreated for 30 min with 1 μM Cl-IB-MECA before superfusion with adenosine, NECA, baclofen, or carbachol; only the responses to adenosine and NECA were inhibited (**$p < 0.001$).

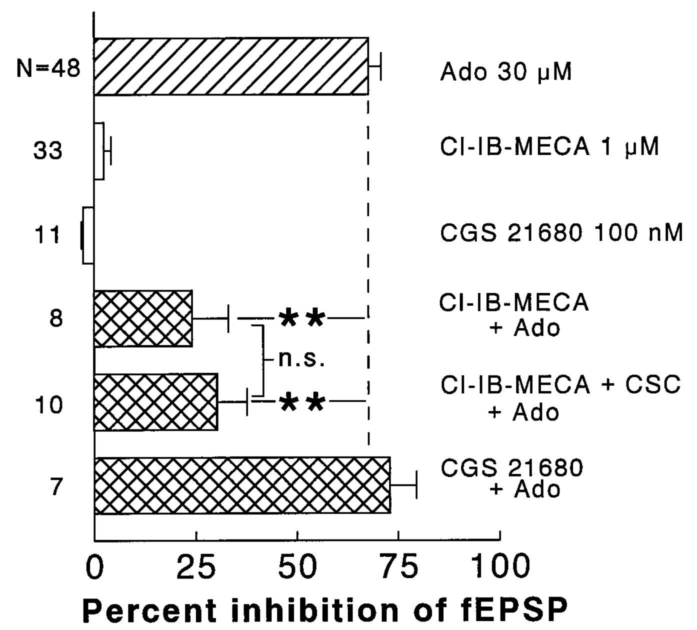

Figure 6. Effects of selective adenosine receptor agonists and antagonists on fEPSP responses. Slices were superfused with the indicated drugs alone (*top three bars*) or pretreated with the indicated drugs and then tested with 30 μM adenosine (*cross-hatched bars*). Each bar shows the mean ± SEM inhibition of the fEPSP response, and the number of slices tested is shown to the *left* of each bar. Neither the selective A_3 agonist (Cl-IB-MECA) nor the A_{2a} agonist (CGS 21680) had a significant effect on the fEPSP response. Pretreatment with 1 μM Cl-IB-MECA significantly attenuated the adenosine response, and this effect was not blocked by the selective A_{2a} receptor antagonist CSC (1 μM). On the other hand, pretreatment with the A_{2a} agonist CGS 21680 (100 nM) had no significant effect on the adenosine response.

had no effect on subsequent responses to 30 μM adenosine (Fig. 6). Second, when slices were pretreated with the A_{2a} selective antagonist CSC at a concentration 20–200 times its k_d for the A_{2a} receptor (1–10 μM), this had no significant effect on the Cl-IB-MECA inhibition of the adenosine response (Fig. 6). The possibility that the A_{2b} receptor might be involved could not be directly ruled out because of the lack of selective A_{2b} agonists or antagonists, but previous experiments have indicated that Cl-IB-MECA has only very weak agonist effects at this receptor ($EC_{50} > 100$ μM) (A. P. IJzerman, unpublished observations).

There are several ways in which activation of A_3 receptors by Cl-IB-MECA might inhibit responses mediated via adenosine A_1 receptors. If the ultimate target of A_3 receptor activation is the G-protein(s) that mediates the A_1 response, then the antagonism might be noncompetitive; on the other hand, activation of a kinase and phosphorylation of the A_1 receptor might simply reduce the affinity of adenosine for the receptor. Therefore, dose–response curves for adenosine were determined under control conditions and in the presence of 1 μM Cl-IB-MECA. The antagonism that was observed under these conditions appeared to be competitive in the sense that the maximal response to adenosine (normally 95–100% inhibition of the fEPSP response) was not reduced by pretreatment with Cl-IB-MECA (Fig. 7). To demonstrate further that this was the case, several slices were incubated with saturating concentrations of Cl-IB-MECA (nominally 100 μM, in nonsuperfused slices; see Materials and Methods) and then tested with adenosine. In every case, adenosine was still able to elicit a 95–100% inhibition of the fEPSP response (Fig. 8). The potency of adenosine in the presence of 100 μM Cl-IB-MECA did not appear to be significantly reduced when compared with its potency in 1 μM Cl-IB-MECA, suggesting that the latter concentration produced an essentially maximal A_3 response.

The present results suggest that responses to low concentrations of adenosine might be reduced if they were preceded by exposure of the slice to high concentrations of adenosine. Therefore, slices were tested with an approximate EC_{50} concentration of adenosine (20 μM), superfused briefly with 1 mM adenosine, and then retested with 20 μM adenosine. As illustrated in Figure 9, treatment with 1 mM adenosine was often sufficient to completely abolish the inhibitory effect of 20 μM adenosine on the fEPSP response. Similar tests on 16 other slices showed a similar loss of responsivity to low concentrations of adenosine after superfusion with a high concentration (mean initial response to adenosine = 47 ± 7% inhibition of the fEPSP response vs 11 ± 4% after 1 mM adenosine; $n = 16, p < 0.0001$).

DISCUSSION

Although the adenosine A_3 receptor is expressed in brain, there have been no previous reports indicating how activation of this receptor might affect neuronal activity. Behavioral studies have indicated that A_3 receptor agonists have depressant effects on locomotor activity (Jacobson et al., 1993), and the relative lack of sensitivity of these responses to A_1 and A_{2a} receptor antagonists is consistent with mediation via A_3 receptors. Other studies have demonstrated that A_3 agonists have a deleterious effect on survival after an ischemic challenge (von Lubitz et al., 1994) and result in increased hippocampal damage after recovery from ischemia, but the mechanisms involved are unclear. The present study has demonstrated that an important action of A_3 agonists may be

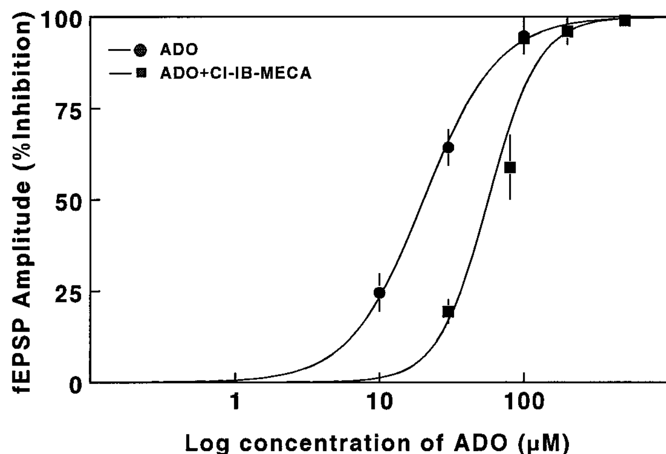

Figure 7. Effect of Cl-IB-MECA on the adenosine dose–response curve. Mean dose–response curves are shown for adenosine alone, and adenosine + Cl-IB-MECA (1 μM). Each *point* is the mean ± SEM response of at least five slices tested with the corresponding concentration of adenosine. EC_{50} values for the two conditions were 26 μM and 66 μM. The effect of Cl-IB-MECA on the EC_{50} value was statistically significant ($p < 0.001$), but the slopes of the corresponding dose–response curves (1.7, 1.6) were not significantly different. Note that the maximal effect of adenosine, which is normally a 95–100% inhibition of the fEPSP response, was not affected by Cl-IB-MECA.

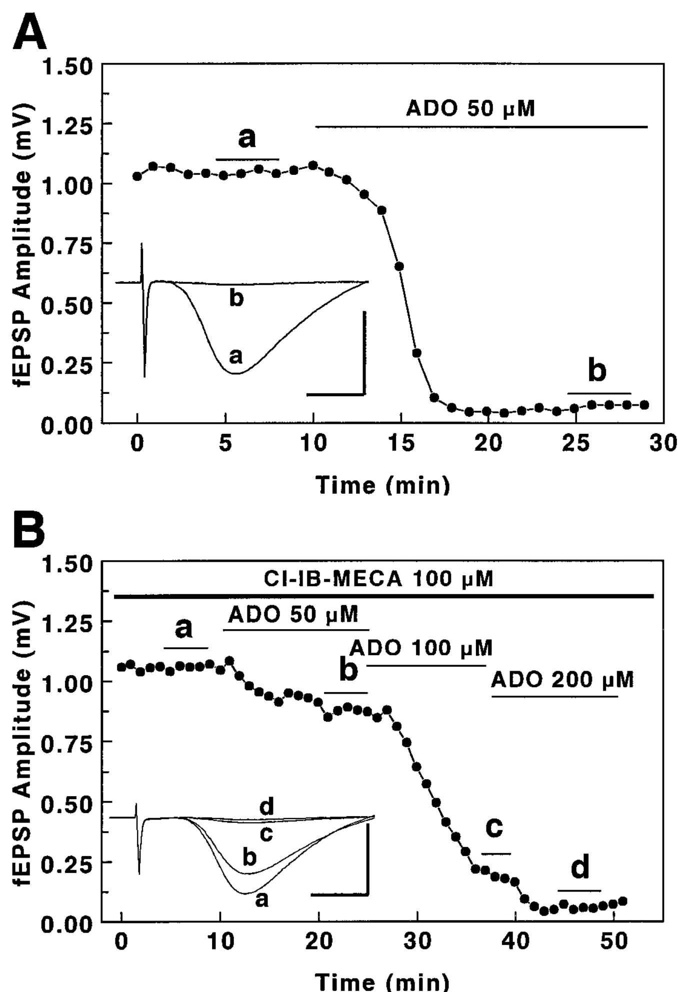

Figure 8. A high concentration of Cl-IB-MECA reduces the potency of adenosine but not its maximal effect. Slices were incubated in a nonsuperfused slice chamber and were treated with adenosine alone (*A*) or pretreated with 100 μM Cl-IB-MECA and then tested with adenosine (*B*). Adenosine was added sequentially to achieve the indicated concentrations, but because the chamber was not superfused, washout was not possible with this experimental protocol. *A* illustrates an experiment with a control slice, which was pretreated with DMSO for 40 min before the beginning of the record (DMSO was used initially to dissolve the Cl-IB-MECA), and then adenosine was added; a concentration of 50 μM essentially eliminated the fEPSP response. In *B*, the slice was incubated in 100 μM Cl-IB-MECA for 40 min (data not shown), then tested with increasing concentrations of adenosine. As in *A*, the fEPSP response could be completely inhibited, but the concentration of adenosine required was approximately fourfold higher in the presence of Cl-IB-MECA. The *inset* responses are signal averaged fEPSPs obtained during the periods indicated by the *lettered bars*. Calibration: 1 mV, 4 msec.

to reduce the sensitivity of adenosine receptors of the A_1 subtype. If this is the primary action of the A_3 receptor, this would be a somewhat novel function for this receptor, but there are reasons why this may be particularly important insofar as the adenosine A_1 receptor is concerned. Previous studies have demonstrated that presynaptic A_1 receptors in brain are tonically activated by endogenous concentrations of adenosine (Dunwiddie and Hoffer, 1980; Dunwiddie and Diao, 1994), and antagonism of this tonic inhibitory action underlies the excitatory effects of adenosine antagonists on behavior (Snyder et al., 1981; Katims et al., 1983). We have also observed that even in the continuous presence of relatively high concentrations of an A_1 agonist, there is no appreciable desensitization of the A_1 response (Dunwiddie and Fredholm, 1984). Because of this, adenosine is able to exert a tonic inhibitory effect on brain activity, an effect that would be difficult to maintain if A_1 receptors desensitized. However, the lack of homologous desensitization of the A_1 receptor raises the issue of how adenosine A_1 receptor sensitivity is regulated; the present findings suggest that this may be the role of the A_3 receptor. Because the A_3 receptor has a lower affinity for adenosine than does the A_1, normal brain concentrations of adenosine (estimated to be 150–200 nM) (Dunwiddie and Diao, 1994) are unlikely to activate a significant fraction of A_3 receptors. However, under conditions in which brain adenosine concentrations rise (hypoxia, ischemia, seizures, etc.), activation of A_3 receptors may lead to a heterologous desensitization of the A_1 response. This hypothesis could explain why A_3 receptor agonists exacerbate the effects of ischemia. A_1 receptor activation has been shown to have cerebroprotective effects in ischemia (Daval et al., 1991; Rudolphi et al., 1992), whereas antagonism of A_1 receptors by competitive receptor antagonists such as theophylline or caffeine (Rudolphi et al., 1987, 1992) worsens the outcome, presumably by blocking the actions of endogenous adenosine. Thus, if activation of A_3 receptors by an agonist such as Cl-IB-MECA reduces the sensitivity of A_1 receptors, it would also be expected to reduce the protective effects of endogenous adenosine, whereas antagonists such as MRS 1191, which in the present study selectively antagonized A_3 receptors, might be neuroprotective.

The mechanism by which Cl-IB-MECA antagonizes A_1 receptor-mediated responses is unclear. One possible mechanism would be a direct receptor antagonism, which could occur if Cl-IB-MECA were a weak partial agonist or antagonist at A_1 receptors. However, if this were the case, one would predict that the high concentration of Cl-IB-MECA tested in Figure 8 should have shifted the adenosine dose–response curve by a factor of ~20-200 (based on the effect of 1 μM Cl-IB-MECA illustrated in Fig. 7), but the observed shift was approximately fourfold, which is not consistent with a competitive interaction. The observation

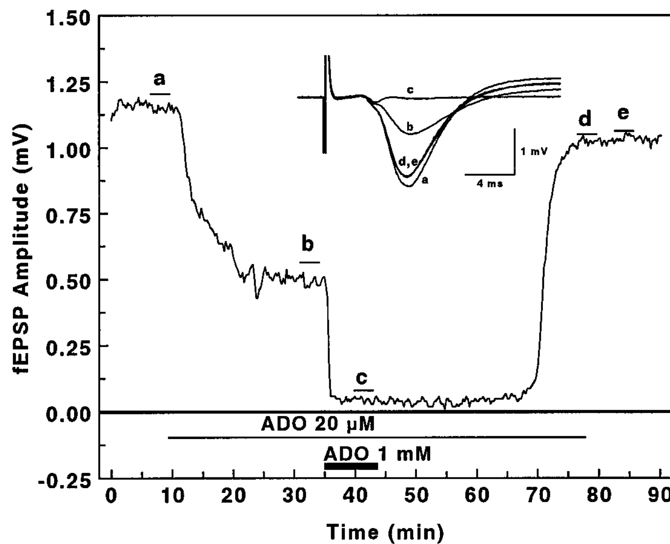

Figure 9. Desensitization induced by high concentrations of adenosine. A hippocampal slice was superfused initially with 20 μM adenosine, then the concentration was briefly increased to 1 mM, then reduced back to 20 μM, as indicated by the lines at the *bottom*. Signal averages of evoked fEPSPs in the *inset* correspond to responses evoked during the periods indicated by *lettered line segments*. The fact that the response recovered to a much higher level in 20 μM adenosine (d > b) and showed no change at all when the 20 μM adenosine superfusion was ended suggested that the inhibitory effects of this concentration of adenosine had been completely lost (a→b = 56% inhibition, vs e→d = 1% inhibition). The maximal response to adenosine did not appear to be affected during the 1 mM adenosine superfusion. The slight decrease in the baseline from *a* to *e* was not consistently observed.

that the selective A_3 receptor antagonist MRS 1191 blocked the effects of Cl-IB-MECA, while having no direct effect either on the A_1 receptor or on responses to adenosine, provides even more compelling evidence that Cl-IB-MECA does not interact directly with the A_1 receptor to reduce the response to adenosine.

Activation of A_3 receptors has been linked to both inhibition of adenylyl cyclase (Zhou et al., 1992) as well as to activation of phospholipase C (Ramkumar et al., 1994). Although either mechanism could be involved, the latter response, and presumably the concomitant activation of PKC, appears to be the principal transduction mechanism in brain (Abbracchio et al., 1995). We have observed that chelerythrine, which is an inhibitor of PKC, was able to reverse the effects of Cl-IB-MECA (data not shown), although the interpretation of these results is unclear because of the fact that PKC inhibitors alone alter A_1 receptor sensitivity. Nevertheless, a mechanism involving PKC would also be consistent with previous studies that have shown that activation of PKC by phorbol esters, or muscarinic receptor agonists, can antagonize the presynaptic effects of adenosine in hippocampus (Worley et al., 1987; Thompson et al., 1992, 1993). If PKC is involved, the substrate that it phosphorylates is unclear, but presumably the A_1 receptor itself, and the G-protein(s) that mediate A_1 responses are likely candidates.

An interesting aspect of the antagonism of A_1 responses by Cl-IB-MECA is that it was specific for the A_1 receptor. Presynaptic $GABA_B$ and muscarinic cholinergic receptors, which have inhibitory effects on excitatory transmission that parallel those of A_1 agonists, were unaffected by Cl-IB-MECA. This is unlike the situation that has been reported previously with muscarinic receptors and phorbol esters, in which both $GABA_B$ and adenosine responses were inhibited (Worley et al., 1987; Thompson and Gahwiler, 1992; Thompson et al., 1992, 1993). Our observations would argue against an action of Cl-IB-MECA on a common mechanism (e.g., presynaptic Ca^{2+} channels), and would suggest that the cellular processes linked to A_3 receptor activation exert their effect either directly at the receptor level or on some other aspect of the transduction mechanism that is unique to the A_1 receptor. This conclusion is consistent with a previous report that suggests that the A_1 and $GABA_B$ receptors modulate transmission through somewhat different mechanisms (Klapstein and Colmers, 1992).

A final issue that is clarified by the present studies has to do with the rebound excitability that has been occasionally reported after adenosine treatment in various systems. For example, Nishimura et al. have reported that in guinea pig hippocampus, treatment with relatively high concentrations of adenosine (50 μM) leads to a postinhibitory rebound excitation that is manifested when adenosine is washed out of the brain slice; furthermore, this effect is antagonized by three different inhibitors of PKC (Nishimura et al., 1992). Because hippocampal responses are tonically inhibited by endogenous adenosine (Dunwiddie and Hoffer, 1980), we would hypothesize that activation of A_3 receptors, and the ensuing heterologous desensitization of A_1 receptors, should lead to a loss of this tonic inhibition, which would be seen as the postexcitatory rebound. The mediation of this process by PKC would be consistent with this proposed mechanism involving A_3 receptors.

The present studies have demonstrated that selective activation or antagonism of adenosine A_3 receptors alone has no direct effect on synaptic transmission or synaptic plasticity in the CA1 region of rat hippocampus. However, activation of these receptors reduces the sensitivity of presynaptic A_1 receptors that inhibit glutamate release in this preparation, whereas responses to other presynaptic modulators at these synapses are unaffected. Based on these results, we hypothesize that an important role of A_3 receptors in brain may be to regulate the level of sensitivity of A_1 receptors, which normally are tonically activated by low concentrations of endogenous adenosine. Antagonizing A_3 receptors might be predicted to enhance the cerebroprotective effects of endogenous adenosine during periods of metabolic stress (e.g., during ischemia or seizures) by preventing the uncoupling of A_1 receptors by the high concentrations of adenosine that are formed under these conditions.

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