Adenosine Modulation of Calcium Currents and Presynaptic Inhibition of GABA Release in Suprachiasmatic and Arcuate Nucleus Neurons

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Chen, Gong and Anthony N. van den Pol. Adenosine modulation of calcium currents and presynaptic inhibition of GABA release in suprachiasmatic and arcuate nucleus neurons. J. Neurophysiol. 77: 3035–3047, 1997. Adenosine modulation of calcium channel currents and synaptic γ-aminobutyric acid (GABA) release was investigated with whole cell voltage-clamp recordings in rat suprachiasmatic nucleus (SCN) and arcuate nucleus cultures (n = 94). In SCN cultures, ~70% of the neurons showed a reversible inhibition of whole cell barium currents on the application of adenosine or its analogues. Adenosine at 1 μM reduced the amplitude of the barium currents by ~27%. In contrast to the significant reduction in the amplitude, the rising and decaying phases of the barium currents, and the inverted bell shape of the current-voltage curve of the barium currents, were not changed by adenosine. The adenosine A1 receptor agonist N6-cyclopentyladenosine (CPA; 100 nM) and the adenosine A2 receptor agonist N6-[2-(3,5-dimethoxyphenyl)-ethyl]adenosine (DPMA; 100 nM) inhibited the calcium currents by 21% and 16%, respectively, in SCN neurons, indicating both A1 and A2 receptor actions. The A1 receptor antagonist 8-cyclopentyl-1,3-dipropylxanthine (100 nM) significantly reduced the effect of CPA but did not change the effect of DPMA on the barium currents. In the presence of tetrodotoxin to block action potentials, the frequency, but not the amplitude, of miniature inhibitory postsynaptic currents was significantly reduced (46%) by 1 μM adenosine, suggesting a presynaptic mechanism of adenosine action. In support of this suggestion, the postsynaptic GABA receptor responses were not influenced by 1 μM adenosine in the majority of SCN neurons. Most solitary self-innervating SCN neurons in microisland cultures were GABAergic. In these cells, the evoked autaptic GABA release (inhibitory postsynaptic current) was significantly inhibited by adenosine (37%), CPA (27%), and DPMA (28%), indicating that both A1 and A2 receptors were present in presynaptic axons. Similar to the effect in SCN neurons, adenosine inhibited both barium currents and GABA release in arcuate neurons. The reduction of whole cell barium currents by adenosine (1 μM), CPA (100 nM), and DPMA (100 nM) was 24, 17, and 19%, respectively. In solitary self-innervating arcuate neurons, adenosine inhibited the evoked GABA release (inhibitory postsynaptic current) by ~48%. We conclude that both adenosine A1 and A2 receptors are present in the SCN and arcuate nucleus of the hypothalamus. Adenosine inhibits calcium currents and presynaptically reduces inhibitory GABA neurotransmission.

INTRODUCTION

Adenosine is a potent neuroactive substance widely distributed throughout the CNS (Dunwiddie 1985; Greene and Haas 1991). Cumulative evidence indicates that both exogenous and endogenously released adenosine substantially suppresses excitatory neurotransmitter release in the spinal cord and various brain regions, particularly in the hippocampus (Alzheimer et al. 1993; Lambert and Teyler 1991; Li and Perl 1994; Manzoni et al. 1994; Mitchell et al. 1993; Prince and Stevens 1992; Proctor and Dunwiddie 1987; Thompson et al. 1992; Yoon and Rothman 1991). In a previous study with digital calcium imaging, we found that adenosine depressed the glutamate-mediated calcium rises in hypothalamic neurons (Obrietan et al. 1995). Limited data are found on the adenosine inhibition of inhibitory neurotransmitters (Kirk and Richardson 1994; Ulrich and Huguenard 1995; Umemiya and Berger 1994) and this has not been examined in hypothalamic neurons. Adenosine inhibition of neurotransmission has been suggested to be mainly through a presynaptic mechanism (Alzheimer et al. 1993; Li and Perl 1994; Manzoni et al. 1994; Thompson et al. 1992), possibly mediated by an inhibition of calcium channels in the presynaptic nerve terminals (Scholz and Miller 1991; Wu and Saggau 1994; Yawo and Chuhma 1993) or an inhibition of the release apparatus independent of the inhibition of calcium influx (Scanziani et al. 1992; Scholz and Miller 1992).

Adenosine receptors are divided into two major subtypes, the A1 and A2 receptors, although molecular cloning has recently demonstrated some other subtypes (Collis and Hourani 1993; Fastbom et al. 1987; Linden et al. 1991; Olah and Stiles 1995). The activation of adenosine A1 receptors often results in the decrease of intracellular cyclic AMP (cAMP) levels; in contrast, the activation of A2 receptors has been reported to lead to an increase of the intracellular cAMP concentration (Dunwiddie 1985; Olah and Stiles 1995). A1 receptors are found throughout the brain (Fastbom et al. 1987; Mahan et al. 1991; Reppert et al. 1991). The inhibition of calcium channels and transmitter release in the CNS is primarily mediated through the activation of A1 receptors (Alzheimer et al. 1993; Manzoni et al. 1994; Mitchell et al. 1993; Mogul et al. 1993; Ulrich and Huguenard 1995; Umemiya and Berger 1994; Wu and Saggau 1994; Yawo and Chuhma 1993; Zhu and Ikeda 1993). The distribution of A2 receptors is more restricted to certain brain regions (Meng et al. 1994; Nonaka et al. 1994), and A2 receptor activation can either inhibit or enhance barium currents and transmitter release (Kirk and Richardson 1994; Mogul et al. 1993; O’Regan et al. 1992a,b; Sebastiao and Ribeiro 1992; Umemiya and Berger 1994).

Accumulative evidence demonstrates a circadian fluctuation of both adenosine and adenosine receptors in mammalian brain (Chagoya de Sanchez 1995; Florio et al. 1991; Virus et al. 1984). However, the effect of adenosine in the suprachiasmatic nucleus (SCN) of the hypothalamus, the control center of mammalian circadian rhythms, has not been explored before. Several lines of evidence suggest that adenosine is present and plays a functional role in the hypothala-
mus. Although only a low density of adenosine receptors has been reported in the hypothalamus (Fastbom et al. 1987; Reppert et al. 1991), the highest level of adenosine uptake sites and adenosine deaminase were found in the hypothalamus (Nagy et al. 1984, 1985). Adenosine A1 receptor binding in the rat hypothalamus was increased by chronic stress (Anderson et al. 1988). Administration of adenosine into the preoptic area of the rat hypothalamus influenced sleep and temperature (Ticho and Radulovacki 1991). Adenosine stimulated the secretion of dopamine by hypothalamic neurons (Porter et al. 1995). In a previous study, we showed that adenosine antagonists raised the level of synaptic activity mediated calcium transients of hypothalamic neurons in culture, suggesting that adenosine was actively secreted by hypothalamic cells (Obrietan et al. 1995).

The purpose of the present study was to examine the effect of adenosine on inhibitory $\gamma$-aminobutyric acid (GABA) neurotransmission in hypothalamic neurons from the SCN and arcuate nucleus; the mechanisms of adenosine action have not been previously studied in these regions at the acute level. Whole cell voltage-clamp recordings were made with the use of EPC-7 amplifiers. Patch pipettes were pulled from thin-wall borosilicate glass (World Precision Instrument) with a micropipette puller (Narishige USA) under computer control. The cell suspension was centrifuged at 13,000 rpm for 20 min to remove debris. The cell suspension was centrifu-
ged with the electrode solution. The tight seal resistance was polynucleotides and adenosine deaminase were found in the SCN and arcuate nucleus, respectively (Ralph et al. 1995; Sharma et al. 1990). Usually a solitary single neuron in a microisland was chosen for experimentation.

The culture medium contained minimal essential medium (Gibco) supplemented with 10% fetal calf serum (Hyclone) and serum extender (Collaborative), 100 U/ml penicillin/streptomycin, and 6 g/l glucose. The cultured cells were maintained in an incubator at 37°C and 5% CO2, and used within 5 wk. Multicellular cultures were fed twice weekly, and microcultures were fed once weekly. Cytosine arabinoside (5 µM) was added to cultures after 2–5 days of plating to inhibit the proliferation of astrocytes.

**Electrophysiological recording**

Whole cell voltage-clamp recordings were made with the use of a List EPC-7 amplifier. Patch pipettes were pulled from thin-wall borosilicate glass (World Precision Instrument) with the use of a Narishige vertical puller. The holder was attached to a microinjector (Narishige USA) under computer control. The recording chamber was changed by bath solution at a rate of 1.5 ml/min. Drugs were applied primarily through a series of glass fluid pipes (400 µm ID) fed by gravity, and sometimes through bath perfusion. Experiments were performed at room temperature (~22°C). The data are reported as means ± SE, and the paired Student’s t-test was used in statistical analysis except as otherwise designated.

The standard bath solution contained (in mM) 155 NaCl, 2.5 KCl, 2 CaCl2, 1 MgCl2, 10 N-2-hydroxyethylpiperazine-N'-2-ethanesulfonic acid (HEPES), and 10 glucose, pH adjusted to 7.3 with NaOH. The electrode solution contained (in mM) 145 KMeSO4 (or 100 KMeSO4, 45 KCl), 1 MgCl2, 10 HEPES, 2 K2-β-(aminophenoxy)-N,N,N',N'-tetraacetic acid (BAPTA), 4 Mg-ATP, and 0.5 Na2-guanosine 5'-triphosphate (GTP), pH adjusted to 7.3 with KOH. An electrode solution with a high concentration of Cl− was used in some experiments to obtain a large inward current during recording of GABA responses. For recording calcium channel currents, barium was used as the current carrier. The bath solution contained 114.5 mM NaCl, 40 mM tetraethylammonium hydrochloride (TEA-Cl), 2.5 mM KCl, 5 mM BaCl2, 10 mM HEPES, 10 mM glucose, and 1 mM tetrodotoxin (TTX), pH adjusted to 7.3 with NaOH. The electrode solution contained (in mM) 145 CsMeSO4, 5 Cs2-BAPTA, 10 HEPES, 4 Mg-ATP, and 0.5 Na2-GTP, pH adjusted to 7.3 with CsOH. A protocol (4 subpulses with 1/4 amplitude and opposite direction of the testing pulse) was used to subtract the leak and capacitance current with poly-D-lysine (0.3 ± 0.5 mg/ml). For microcultures containing solitary neurons, the culture dish was first coated with a thin layer of agarose through a microinjection pipette (40–80 µm tip diameter) attached to a syringe (Furshpan et al. 1986). The distance between microdots was ~1–1.2 mm, and the diameter of the microdots ranged from 100 to 500 µm. The microdots of poly-D-lysine served as an adhesive substrate for neurons. The cell suspension was first diluted and then plated in a very low density in the culture dishes (2,000–5,000 cells/cm2). Cells not attached to the microislands of poly-D-lysine died. Typically, in a single polylysine microisland, one or more neurons were found together with one or more glial cells. The isolated neurons in the microislands tended to be self innervating after 2 wk in culture (Furshpan et al. 1986; Segal and Furshpan 1990).

**Methods**

**Cell culture**

The culture of hypothalamic neurons was similar to methods previously described (Chen et al. 1995, 1996) with some modifications. Sprague-Dawley rats (1 day postnatal) were anesthetized with Nembutal and the brain was dissected out. The hypothalamic area was then dissected out from the optic chiasm to the mammillary area and cut coronally into 400-µm hypothalamic slices. The SCN was punched out or microsurgically dissected from the slice under microscope observation. The region of the SCN could be clearly seen with anatomic landmarks including the optic chiasm and third ventricle. In parallel, caudally to the SCN, the arcuate nuclei were removed from the slice in which the third ventricle extended laterally over the median eminence. The tiny tissue blocks were then incubated for 30–40 min in an enzyme solution containing 10 U/ml papain, 0.5 mM EDTA, 1.5 mM CaCl2, and 0.2 mg/ml l-cysteine. The tissue samples were washed twice with culture medium after enzyme treatment and triturated mechanically extended laterally over the median eminence. The tiny tissue blocks were then dissected out from the optic chiasm to the mammillary area and cut coronally into 400-µm hypothalamic slices. The SCN was punched out or microsurgically dissected from the slice under microscope observation. The region of the SCN could be clearly seen with anatomic landmarks including the optic chiasm and third ventricle. In parallel, caudally to the SCN, the arcuate nuclei were removed from the slice in which the third ventricle extended laterally over the median eminence. The tiny tissue blocks were then incubated for 30–40 min in an enzyme solution containing 10 U/ml papain, 0.5 mM EDTA, 1.5 mM CaCl2, and 0.2 mg/ml l-cysteine. The tissue samples were washed twice with culture medium after enzyme treatment and triturated mechanically extended laterally over the median eminence. The tiny tissue blocks were then incubated for 30–40 min in an enzyme solution containing 10 U/ml papain, 0.5 mM EDTA, 1.5 mM CaCl2, and 0.2 mg/ml l-cysteine. The tissue samples were washed twice with culture medium after enzyme treatment and triturated mechanically extended laterally over the median eminence. The tiny tissue blocks were then incubated for 30–40 min in an enzyme solution containing 10 U/ml papain, 0.5 mM EDTA, 1.5 mM CaCl2, and 0.2 mg/ml l-cysteine. The tissue samples were washed twice with culture medium after enzyme treatment and triturated mechanically extended laterally over the median eminence.
from Research Biochemicals International. CNQX, CPA, DPMA, and DPCPX were dissolved in dimethyl sulfoxide (DMSO) to obtain the stock solution. The final DMSO concentration for CNQX was 0.1%, and 0.01% for CPA, DPMA, and DPCPX. The very low concentration of DMSO had no effect on barium currents or synaptic release. Adenosine, CPA, DPMA, and DPCPX were made fresh before each experiment.

**RESULTS**

**Suprachiasmatic nucleus**

A total of 64 SCN neurons was examined for the effect of adenosine on whole cell barium currents, spontaneous GABA release, evoked GABA release, and postsynaptic GABA receptor responses under whole cell voltage clamp. Consistent with previous studies showing that GABAergic neurons dominate the SCN, we found that the GABAergic spontaneous activity dominated in the multicellular cultures and that ~90% of single self-innervating neurons showed evoked GABA release in microisland cultures. Although receptor binding assays showed only a low density of adenosine receptors in the hypothalamus, we found that adenosine inhibited barium currents and GABA release in more than half the SCN neurons tested, consistent with our previous observation with digital imaging that adenosine depressed intracellular calcium concentration in the hypothalamus (Obrietan et al. 1995).

**ADENOSINE INHIBITS BARIUM CURRENTS.** Whole cell barium currents were evoked at a holding potential (HP) of −80 mV and a testing potential (TP) of 0 mV for a duration of 40 ms. Consecutive voltage pulses of the same depolarization (from −80 to 0 mV) were repeated at an interval of 20 s to evoke barium currents. After stable barium currents were obtained, adenosine was applied for 1–3 min to examine its effect on barium currents. In 9 of 13 SCN neurons, we observed an inhibition of barium currents on the adenosine application (1 μM). The other four neurons showed no change. The maximal inhibition was 56%. Figure 1 shows a typical example of the adenosine inhibition on barium currents. In Fig. 1A, adenosine (labeled ADO) depressed the amplitude of barium currents by ~23%. In contrast to the reduction of amplitude, the rising and decaying phases of the barium current during the presence of adenosine were similar to that of the control. The barium currents recovered fully after washout of adenosine. Figure 1B illustrates the effect of adenosine on the current-voltage (I-V) curves of the barium currents. The I-V curve was generated by a series of depolarizing TPs ranging from −50 to +90 mV at an HP of −80 mV. The barium currents were detectable at −40 mV, peaked at 0 mV, and reversed at about +60 mV. Adenosine inhibited the amplitude of the barium currents throughout the tested voltage ranges, suggesting no voltage dependence of the adenosine inhibition. The inverted bell shape of the I-V curve was not changed by adenosine. Cadmium (30

![Fig. 1](http://jn.physiology.org/)

**FIG. 1.** Adenosine inhibits whole cell barium currents in suprachiasmatic nucleus (SCN) neurons. **A**: voltage-activated barium current records before, during, and after application of 1 μM adenosine (ADO). Holding potential (HP) = −80 mV, testing potential (TP) = 0 mV. **B**: current-voltage (I-V) curves of barium currents before and during application of adenosine. Peak point and reversal potential were not changed by adenosine. **C**: cadmium (30 μM) abolished barium currents. **D**: line graph showing relative amount of inhibition of barium currents ($I_{Ba}$) by adenosine and cadmium in same neuron.
μM), a potent calcium channel blocker, almost totally blocked the whole cell barium currents (Fig. 1C), confirming that the currents were carried through calcium channels. Figure 1D compares the effects of adenosine and cadmium in the same neuron, in which adenosine partially inhibited the barium currents and cadmium completely blocked the barium currents. Both inhibitions were reversible.

ADENOSINE A1 AND A2 RECEPTOR AGONISTS INHIBIT BARIUM CURRENTS. There are two adenosine receptors subtypes, A1 and A2 receptors. CPA is a specific A1 receptor agonist, and DPMA is a specific A2 receptor agonist. To explore through which receptor subtype adenosine inhibits barium currents, we tested the effects of both A1 and A2 agonists and found that each inhibited the barium currents. Figure 2A demonstrates that the A2 agonist DPMA (100 nM) inhibited the barium currents. The two recording traces (i and ii) correspond to the two points in the I-V curves. The amplitude of the barium currents was reduced by DPMA, whereas the shape of the I-V curve was not changed. Figure 2B shows that the adenosine A1 receptor agonist CPA, similar to DPMA in Fig. 2A, also inhibited the barium currents in the same neuron, suggesting a coexistence of A1 and A2 receptors in the SCN. We also observed an inhibition of adenosine (1 μM) on barium currents at a more positive HP (HP = −40 mV, TP = 0 mV, data not shown).

To exclude the possibility that the A2 agonist cross-activated A1 receptors, we combined the A2 agonist with DPCPX, a specific antagonist for A1 receptors. Figure 3A demonstrates that the application of either DPMA or CPA to the same neuron inhibited the barium currents, suggesting the coexistence of A1 and A2 receptors in this neuron. In the presence of DPCPX, application of the A2 agonist DPMA to the same neuron still inhibited the barium current, whereas the inhibiting effect of A1 agonist CPA was abolished (Fig. 3B), confirming an inhibition of barium currents through A2 receptor activation, independent of A1 receptors. Figure 3C summarizes the data in SCN neurons on the reduction of barium currents by cadmium, adenosine, and its analogues. Cadmium (30 μM) inhibited barium currents by 94.6 ± 1.2% (mean ± SE; n = 5). Adenosine (1 μM) inhibited barium currents to ~70% of the control, and its analogue CPA (100 nM), DPMA (100 nM), and DPMA (100 nM) plus DPCPX (100 nM) inhibited barium currents to ~80% of the control. All these inhibitions were statistically significant (P < 0.05, t-test). In contrast, CPA (100 nM) plus DPCPX (100 nM) did not reduce the barium currents to statistical significance (7.5 ± 4.2%, P > 0.2).

ADENOSINE REDUCES THE MINIATURE INHIBITORY POSTSYNAPTIC CURRENT FREQUENCY. The adenosine inhibition of barium currents in SCN neurons led us to investigate whether transmitter release was affected by adenosine in the SCN. In our initial experiments, we observed an inhibitory effect of adenosine (1 μM) on spontaneous release in normal bath solution (n = 4). We then examined the effect of adenosine on miniature inhibitory post synaptic currents (mIPSCs) in the presence of TTX (1 μM) and the glutamate receptor antagonists AP5 (100 μM) and CNQX (10 μM) in the bath solution to prevent action potential dependent transmitter release and occasional excitatory postsynaptic currents. The four consecutive recording traces in Fig. 4A illustrate the control mIPSCs. Adenosine (1 μM) reduced the frequency of the mIPSCs while leaving the amplitude of mIPSCs unchanged (Fig. 4B). CPA (100 nM) plus DPCPX (100 nM) significantly reduced the frequency of spontaneous IPSCs by 45.5 ± 7.8% (P < 0.01). Figure 5 demonstrates the mIPSC amplitude distribution before and during application of adenosine (1 μM). The Kolmogorov-Smirnov test showed no significant change in amplitudes between the two distributions. The median mIPSC amplitude in the control was 17.8 pA; in adenosine-treated cells it was 17.5 pA. The range of mIPSC amplitude was similar in controls (4.5–75.8 pA) and adenosine-treated cells (4.2–74.3 pA).
FIG. 3. Inhibition of barium currents by A2 agonists does not occur through cross-activation of A1 receptors. A: line graph showing that application of DPMA (A2) and CPA (A1) to same neuron both inhibited barium currents. B: in presence of A1 antagonist 8-cyclopentyl-1,3-dipropylxanthine (DPCPX), A2 agonist still inhibited barium currents, whereas A1 agonist inhibition was abolished. C: pooled data showing reduction of barium currents by cadmium and adenosine and its analogues. CPA + DPCPX had no significant effect on barium currents. Other reduction showed statistical significance (P < 0.05).

ADENOSINE HAS LITTLE EFFECT ON POSTSYNAPTIC GABA RECEPTOR RESPONSES. To further examine the effect of adenosine on postsynaptic GABA receptors, GABA (100 μM) was pneumatically puffed onto neurons under computer control to evoke postsynaptic responses. TTX (1 μM) and the glutamate receptor antagonists AP5 (100 μM) and CNQX (10 μM) were present in the bath solution to block action potential dependent synaptic release and glutamate actions. Twelve neurons were examined. Of these, 10 showed no changes in the GABA responses on the application of 1 μM adenosine. Figure 6, A1–A3, shows a typical example of the 10 neurons. Adenosine had little effect on the currents induced by GABA puff (50 ms, 5 psi). As for the remaining two neurons, one showed an increase and one showed a decrease of the GABA receptor response on the application of adenosine. The bar graph of Fig. 6B summarizes the results of all 12 neurons. When the control GABA responses were normalized to 100%, the mean GABA response in the presence of 1 μM adenosine was 104.8 ± 3.2%, not significantly different from control (P > 0.5).

ADENOSINE INHIBITS EVOCED GABA RELEASE IN SELF-INNERVATING SCN NEURONS. Central neurons not only form synapses with other neurons, but also can synapse on themselves (Lubke et al. 1996). This type of synapse (autapse) can be induced under conditions in vitro that restrict axonal growth away from the cell body. In the present study we cultured SCN neurons in low cell density in microislands so that neurons were isolated from each other. After 2 wk of culture, the isolated single neurons formed synapses with themselves, and a voltage pulse in voltage clamp could evoke an action current followed by an evoked IPSC. Figure 7 A1 illustrates such a scenario, in which a short voltage pulse (2 ms, 60 mV) evoked an action current (labeled $I_{Na}$) and an IPSC. The IPSC could be blocked by the GABA receptor antagonist BIC (30 μM; Fig. 7A2). In Fig. 7, B1–B3, the control trace also shows an action current and an IPSC ($B1$).
In the presence of 1 μM adenosine (B2) the evoked IPSC was markedly inhibited, whereas the sodium action current was unchanged, suggesting a presynaptic inhibition of GABA release in the SCN neurons. The inhibition of the evoked IPSC by adenosine was also reversible (B3). Of a total of 26 neurons examined, 14 showed a reduction in the evoked GABA release on the application of adenosine and its analogues. Figure 7C summarizes the results obtained from the 14 neurons. The reduction of IPSC amplitude by adenosine (1 μM), CPA (100 nM), and DPMA (100 nM) was 37.1 ± 6.2%, 27.3 ± 6.6%, and 27.5 ± 4.5%, respectively. All of the reductions reached statistical significance (P < 0.05). The dose-response relationship between the adenosine concentration and the reduction of evoked IPSCs was investigated in six neurons (Fig. 8). Adenosine at 10 μM produced a similar reduction to adenosine at 5 μM, indicating that it was close to the saturating concentration. The data were fitted by a logistic equation

\[ F = \frac{F_{\text{max}}}{1 + (\text{EC}_{50}/[\text{ADO}])^n} \]

with the use of a nonlinear least-squares method where \( F \) is the reduction of the IPSC by adenosine, \( F_{\text{max}} \) is the maximum reduction, and \( n \) is the Hill coefficient. The maximum adenosine effect was ~45% reduction, and the median effective concentration (EC50) was ~0.4 μM. These experiments demonstrate that adenosine exerts a potent inhibition on inhibitory GABA neurotransmission in SCN neurons.

**Arcuate nucleus**

To determine whether the effect of adenosine observed above is unique in the SCN or exists in other regions of the hypothalamus, we extended the experiments to the arcuate nucleus, a part of the hypothalamus that regulates endocrine secretions. We found an effect of adenosine similar to that in the SCN in that adenosine inhibited both barium currents and evoked GABA release in neurons from the arcuate nucleus.

**Barium currents.** In arcuate nucleus cultures, 21 neurons were examined for the effect of adenosine or its analogues on whole cell barium currents. Twelve neurons showed a reduction, whereas the other nine neurons showed no change in the barium currents. The reduction of the barium currents by adenosine (1 μM), CPA (100 nM), and DPMA (100 nM) was 23.5 ± 5.1% (n = 4), 17.1 ± 1.3% (n = 5), and 19.0 ± 4.3% (n = 5), respectively (P < 0.05). A representative experiment is illustrated in Fig. 9. The four
barium current traces in Fig. 9, B1 and B2, labeled i–iv, correspond to the four points in Fig. 9A, left plot. Similar to the SCN cultures, CPA and DPMA both inhibited the barium currents in arcuate neurons, suggesting a coexistence of adenosine A1 and A2 receptors in the arcuate nucleus.

**EVOKED GABA RELEASE.** In contrast to the SCN cultures that were composed primarily of GABAergic neurons, there were both glutamatergic neurons and GABAergic neurons in the arcuate nucleus cultures. In 15 self-innervating arcuate nucleus neurons showing evoked autaptic responses, 11 neurons were GABAergic and 4 neurons were glutamatergic. The evoked IPSC was blocked by $30 \mu M$ BIC, and the excitatory postsynaptic current was blocked by the glutamate receptor antagonist CNQX ($10 \mu M$). Of nine GABAergic neurons examined for the effect of adenosine, five showed a reduction. One glutamatergic neuron also showed a reduction in the excitatory postsynaptic current under adenosine treatment (not shown). Figure 10, A–C, shows an example of adenosine effect on the evoked IPSC. Adenosine at $1 \mu M$ significantly reduced the IPSC amplitude (Fig. 10B), and the inhibition was reversible (Fig. 10C). The bar graph of Fig. 10D shows the average inhibition of the evoked IPSC by adenosine ($1 \mu M$) in the five neurons ($47.8 \pm 11.8\%$, $P < 0.01$). These results demonstrate that adenosine modulates GABA transmission in the arcuate nucleus, similar to its actions in the SCN.

**DISCUSSION**

Our results demonstrate a potent inhibitory action of adenosine in the hypothalamus, showing that a low concentration of adenosine or its analogues inhibits both the neuronal barium currents and synaptic GABA release in cultured SCN and arcuate nucleus neurons. The adenosine inhibition in the hypothalamus is mediated through the activation of both adenosine A1 and A2 receptors.

**Adenosine inhibition of neuronal barium currents**

Adenosine inhibits calcium channels in a number of central neurons, and this inhibition may be critical for transmitter release. The adenosine inhibition of barium currents has been well delineated in the hippocampus. The adenosine analogue 2-chloroadenosine reduced barium currents in rat hippocampal pyramidal neurons, and this reduction was antagonized by the A1 receptor antagonist cyclopentyltheophylline (Scholz and Miller 1991). In guinea pig hippocampal CA3 neurons, activation of A1 receptors inhibited primarily N-type barium currents, whereas activation of A2
FIG. 6. Adenosine has little effect on postsynaptic γ-aminobutyrate (GABA) receptor responses in SCN neurons. A1: control, showing response induced by puff of GABA (200 μM, 5 psi, 50 ms). A 10-mV hyperpolarizing potential was generated in initial trace to monitor recording condition. HP = −60 mV. A2: adenosine (1 μM) had no significant effect on GABA-evoked response. A3: washout of adenosine, showing no changes in GABA-evoked response. B: bar graph showing similar GABA receptor response before and during application of adenosine (1 μM).

receptors significantly increased P-type barium currents (Mogul et al. 1993). In the guinea pig CA1 region of hippocampal slices, adenosine simultaneously inhibited the presynaptic calcium transient and field potentials through activation of A1 receptors, suggesting a relationship between calcium channels and transmitter release (Wu and Saggau 1994). Similar to guinea pig hippocampal CA3 neurons, in the rat brain stem, adenosine also inhibited N-type calcium channels through activation of A1 receptors, but potentiated P-type calcium channels through activation of A2 receptors (Umemiya and Berger 1994).

Our present results, together with our previous study in which calcium digital imaging was used (Obrietan et al. 1995), indicate a potent inhibition of adenosine on barium currents and calcium influx in rat hypothalamic neurons through activation of A1 receptors. In contrast to results in the hippocampus and the brain stem, we found that activation of A2 receptors decreased, not increased, the barium currents of hypothalamic neurons. An inhibitory action of activation of A2 receptors was also found in ischemic rat cerebral cortex (O’Regan et al. 1992b). In the present study, the whole cell barium currents were probably a mixture of several current subtypes (Fisher and Bourque 1995; Koike et al. 1993). We found that adenosine inhibited barium currents in both high (−80 mV) and low (−40 mV) HPs, and in various TPs, suggesting that adenosine inhibition was not dependent on voltage changes.

Adenosine inhibition on synaptic transmitter release

Adenosine inhibited excitatory glutamate neurotransmission in a number of brain areas, including hippocampus (Manzoni et al. 1994; Mitchell et al. 1993; Proctor and Dunwiddie 1987; Scholz and Miller 1992; Thompson et al. 1992; Yoon and Rothman 1991), cerebellar cortex (Kocsis et al. 1984), olfactory cortex (Motley and Collins 1983), thalamus (Ulrich and Huguenard 1995), and spinal cord (Li and Perl 1994). In the hypothalamus, our previous study with calcium digital imaging demonstrated that adenosine released by hypothalamic cells inhibited glutamate-mediated calcium rises (Obrietan et al. 1995). This inhibition was reversed by an adenosine A1 receptor antagonist, and re-
Fig. 7. Adenosine inhibits evoked IPSC in solitary self-innervating SCN neurons. A1: control, showing sodium action current ($I_{Na}$) and following IPSC, evoked by a brief voltage pulse (60 mV, 2 ms). HP = −60 mV. High Cl− concentration in pipette solution. A2: BIC (30 μM) abolished evoked IPSC while leaving $I_{Na}$ barely affected. B1: control of $I_{Na}$ and IPSC in another SCN neuron. B2: adenosine (1 μM) remarkably inhibited evoked IPSC. B3: recovery of IPSC after washout of adenosine. C: pooled data showing that adenosine and its analogue all significantly inhibited evoked IPSCs ($P < 0.05$).

Fig. 8. Dose-response curve for adenosine inhibition on evoked IPSCs. Reduction of IPSCs by 0.1, 0.5, 1, 5, and 10 μM adenosine was 7 ± 3.4% ($P > 0.1$), 26.4 ± 9.9% ($P < 0.05$), 32.5 ± 10.2% ($P < 0.05$), 43.7 ± 9.6% ($P < 0.01$), and 44.2 ± 7.4% ($P < 0.002$), correspondingly. Data were fitted by dotted line with the use of a logistic equation: $F = F_{max}/(1 + (EC_{50}/[ADO])^n)$ (see text). Median effective concentration ($EC_{50}$) = 0.4 μM.
FIG. 9. Adenosine receptor A1 and A2 agonists inhibit whole cell barium currents in arcuate neurons. A: line graph showing that application of both DPMA (100 nM) and CPA (100 nM) to same neuron inhibited barium currents. B1 and B2: barium current traces showing inhibition of DPMA and CPA. Each trace corresponds to a point in line graph in A. HP = −80 mV; TP = 0 mV.

but not GABAergic, nerve terminals was also found in dentate gyrus (Prince and Stevens 1992). In contrast, we demonstrate in the SCN, where GABAergic neurons dominate, that adenosine significantly reduces the frequency but not the amplitude of miniature GABA-mediated IPSCs, suggesting a presynaptic inhibition of GABA neurotransmission. This is corroborated by experiments on self-innervating SCN neurons that show a direct inhibition of adenosine on the evoked GABA release. This inhibition of adenosine on the inhibitory neurotransmission was also found in arcuate nucleus neurons. In contrast to our observation in the hypothalamus that adenosine reduced mIPSCs, in the cerebellum adenosine may inhibit transmitter release presynaptically, but without altering the frequency of mIPSCs (Dittman and Regehr 1996). This suggests that adenosine could reduce GABA release by at least two mechanisms. One mechanism may be mediated by modulation of presynaptic calcium channels, and another may be independent of calcium, possibly a direct modulation on release apparatus. Adenosine has been demonstrated to inhibit GABA release in striatum (Kirk and Richardson 1994) and thalamus (Ulrich and Huguenard 1995), and glycine release in the brain stem (Ümemiya and Berger 1994). Together, these data suggest that, unlike the widespread inhibition of excitatory neurotransmission, adenosine may exert a substantial inhibition on inhibitory neurotransmission in restricted regions of the brain. Because inhibition of inhibitory neurotransmission may increase excitation, this may indicate a complex action of adenosine in the hypothalamus.

Adenosine function in the hypothalamus

There are few data on adenosine action in the hypothalamus compared with those for the hippocampus, in part because receptor binding studies showed a low density of adenosine receptors in the hypothalamus (Fastbom et al. 1987; Mahan et al. 1991; Reppert et al. 1991). However, the present study and our previous work with calcium digital imaging (Obrietan et al. 1995) suggest that the majority of neurons in the hypothalamus possesses adenosine receptors that mediate an inhibition of barium currents or calcium influx. There are a number of possible explanations for this apparent discrepancy. One explanation lies in the difference in detection methods. In previous studies researchers used autoradiographic ligand binding to study the distribution of adenosine receptors in the whole brain, and this method may be relatively insensitive compared with the physiological approach we used here. The relatively low density of adenosine receptors, compared with high densities in other brain regions, does not necessarily indicate a lack of functional adenosine receptors in the hypothalamus. In our experiments with whole cell patch-clamp recordings, we detected changes in barium currents and in the spontaneous and evoked transmitter release in the majority of hypothalamic neurons tested during the presence of adenosine, demonstrating the widespread existence of functional adenosine receptors. Similarly, in our previous study with calcium digital imaging, we also detected a change in the calcium influx mediated by glutamate receptor activation during the presence of adenosine (Obrietan et al. 1995), supporting a participation of adenosine in the modulation of neurotransmission in the hypothalamus. Another possibility is our use of hypothalamic neurons cultured from developing brains, compared with the use of adult preparations in the cited papers. It is possible that adenosine receptors are transiently expressed in a rather high density in the hypothalamus and then gradually reduced during development. However, we did find adenosine re-
FIG. 10. Adenosine inhibits evoked GABA release in solitary self-innervating arcuate neuron. A: control, showing an evoked IPSC. B: adenosine (1 μM) substantially inhibited IPSC ( ). C: recovery of IPSC after washout of adenosine. HP = −60 mV. D: bar graph showing average reduction of evoked IPSC by 1 μM adenosine.

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sponses in mature neurons that had been in culture for >6 wk, suggesting that a whole cell electrophysiological approach is significantly more sensitive than receptor binding assays in detecting adenosine receptors. Whether adenosine receptors show a developmental reduction has not yet been explored. Porter et al. (1995) found that the so-called cytolytic factor that stimulated the secretion of dopamine by neurons of the hypothalamus was actually adenosine. Given our findings, adenosine could increase dopamine secretion by reducing the inhibitory tone exerted by GABAergic axons that terminate on dopaminergic neurons in the arcuate nucleus (van den Pol 1985, 1986). In the hypothalamus, adenosine receptor binding sites were increased by chronic stress, suggesting a possible role of adenosine in stress (Anderson et al. 1988). Other evidence suggesting a potential function of adenosine in the hypothalamus is that the adenosine uptake sites and adenosine deaminase have been found in much higher levels than in other brain regions (Nagy et al. 1984, 1985).

Adenosine receptors are found throughout the brain, and adenosine is implicated in modulating many brain functions, from the biochemical to the behavioral level (Dunwiddie 1985). We show here that adenosine also exerts significant actions on transmitter release in hypothalamic neurons, and that adenosine actions on hypothalamic neurons are different from those found in the more commonly studied hippocampus. We show that adenosine exerts a significant effect in reducing transmitter release at the axon terminal of arcuate nucleus neurons; hypothetically, adenosine could participate in neuroendocrine regulation, including that related to stress, by inhibiting release of neuroactive agents and pituitary tropins in the median eminence, a projection site for many arcuate neurons. The results reported in the present paper, together with our previous work (Obrietan et al. 1995), provide evidence from the cellular level that adenosine may modulate hypothalamic functions through both the depression of intracellular calcium levels and the inhibition of neurotransmitter release. The inhibition of transmitter release may be, at least in part, due to an adenosine-mediated reduction in calcium levels in the presynaptic axons.

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