Adenosine Receptor Expression and Modulation of Ca\textsuperscript{2+} Channels in Rat Striatal Cholinergic Interneurons

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Song, Wen-Jie, Tatiana Tkatch, and D. James Surmeier. Adenosine receptor expression and modulation of Ca\textsuperscript{2+} channels in rat striatal cholinergic interneurons. J. Neurophysiol. 83: 322–332, 2000. Adenosine is a potent regulator of acetylcholine release in the striatum, yet the mechanisms mediating this regulation are largely undefined. To begin to fill this gap, adenosine receptor expression and coupling to voltage-dependent Ca\textsuperscript{2+} channels were studied in cholinergic interneurons by combined whole cell voltage-clamp recording and single-cell reverse transcription–polymerase chain reaction. Cholinergic interneurons were identified by the presence of choline acetyltransferase mRNA. Nearly all of these interneurons (90%, n = 28) expressed detectable levels of A\textsubscript{1} adenosine receptor mRNA. A\textsubscript{2a} and A\textsubscript{2b} receptor mRNAs were less frequently detected. A\textsubscript{2a} receptor mRNA was undetectable. Adenosine rapidly and reversibly reduced N-type Ca\textsuperscript{2+} currents in cholinergic interneurons. The A\textsubscript{1} receptor antagonist 8-cyclopentyl-1,3-dimethylxanthine completely blocked the effect of adenosine. The IC\textsubscript{50} of the A\textsubscript{1} receptor selective agonist 2-chloro-N\textsubscript{6}-cyclopentyladenosine was 45 nM, whereas it was near 30 \mu M for the A\textsubscript{2a} receptor agonist CGS-21680. Dialysis with GDP/BS or brief exposure to the G protein (G\textsubscript{i/o}) alkylating agent 2-chloro-N\textsubscript{6}-ethylmaleimide also blocked the adenosine modulation. The reduction in N-type currents was partially reversed by depolarizing prepulses. A membrane-delimited pathway mediated the modulation, because it was not seen in cell-attached patches when agonist was applied to the bath. Activation of protein kinase C attenuated the adenosine modulation. Taken together, our results argue that activation of A\textsubscript{1} adenosine receptors in cholinergic interneurons reduces N-type Ca\textsuperscript{2+} currents via a membrane-delimited, G\textsubscript{i/o} class G-protein pathway that is regulated by protein kinase C. These observations establish a cellular mechanism by which adenosine may serve to reduce acetylcholine release.

INTRODUCTION

Cholinergic interneurons are key regulators of striatal function (Wooten 1990). Parkinson’s disease, for example, can be treated either by trying to replace the lost dopamine or by antagonizing cholinergic neurotransmission. Cholinergic signaling is normally terminated by the rapid hydrolysis of acetylcholine (ACh) by acetylcholine esterase (AChE). Although cholinergic fibers and terminals are evenly distributed in the rodent striatum (Kemp and Powell 1971; Phelps et al. 1985; cf. Graybiel et al. 1986), AChE is nearly absent from the striosomal compartment (Herkenham and Pert 1981). It is unclear whether other mechanisms are in place to abbreviate cholinergic neurotransmission with this functionally distinctive region (Gerfen 1992; Graybiel 1990).

One potential regulator of cholinergic signaling in the striosomal compartment is adenosine. Several observations are consistent with this possibility. First, 5’-nucleotidase, an ectoenzyme that metabolizes AMP to adenosine, is enriched in striosomes (Schoen and Graybiel 1992). Second, cholinergic interneurons co-release ACh and ATP (Richardson et al. 1987). ATP is rapidly metabolized by ecto-ATPases and ecto-ADPases to AMP in the extracellular space (Brundege and Dunwiddie 1997). Adenosine generated by 5’-nucleotidase metabolism of AMP is capable of modulating neuronal function by activating G-protein–coupled receptors (Palmer and Stiles 1995). To date, four such adenosine receptors have been cloned: A\textsubscript{1}, A\textsubscript{2a}, A\textsubscript{2b}, and A\textsubscript{3} (Fink et al. 1992; Mahan et al. 1991; Stehle et al. 1992; Zhou et al. 1992). Activation of A\textsubscript{1} receptors has been reported to inhibit N-type Ca\textsuperscript{2+} currents, whereas activation of A\textsubscript{2} receptors potentiates P/Q-type Ca\textsuperscript{2+} currents (Gross et al. 1989; Mogul et al. 1993; Mynlieff and Beam 1994; Scholz and Miller 1991; Umemiya and Berger 1994; Zhu and Ikeda 1993). These changes in transmembrane Ca\textsuperscript{2+} flux are thought to underlie the ability of A\textsubscript{1} receptors to inhibit and A\textsubscript{2} receptors to enhance synaptic transmission (Brundege and Dunwiddie 1997).

Similar mechanisms may regulate cholinergic synaptic transmission in the striatum. Pharmacological assays show that activation of A\textsubscript{1} receptors inhibits striatal ACh release (Brown et al. 1990; Jin et al. 1993; Kirkpatrick and Richardson 1993). Although neurons are capable of releasing adenosine itself (Brundege and Dunwiddie 1997), the conversion of released ATP to adenosine in the extracellular space is critical to the A\textsubscript{1} receptor–mediated inhibition of ACh release (Richardson et al. 1987). RNA for A\textsubscript{1} adenosine receptors (the receptors linked to presynaptic inhibition) has been localized to large, presumed cholinergic interneurons in the striatum (Dixon et al. 1996). On the other hand, A\textsubscript{2a} receptor–selective agonists have been reported to either enhance (Brown et al. 1990; Kirkpatrick and Richardson 1993) or have no affect on ACh release (Jin and Fredholm 1997; Jin et al. 1993). Attempts to localize A\textsubscript{2a} receptor mRNA have either concluded that cholinergic interneurons do not express A\textsubscript{2a} receptors (Fink et al. 1992; Schifffmann et al. 1991) or express very low levels (Dixon et al. 1996; Svenningsson et al. 1997).

This study was undertaken to answer two questions. First, what adenosine receptors do identified striatal cholinergic interneurons express? Previous attempts to answer this question have relied on relatively insensitive in situ hybridization techniques and have failed to unequivocally identify the transmitter phenotype of the neurons examined. To overcome these limitations, single-cell, reverse transcription-polymerase chain re-

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acetylcholine. ATP released from synaptic terminals may serve to reduce current rundown. All reagents were obtained from Sigma except for a low metals grade sulfuric acid (Fluka, Ronkowa, NY). Recordings were obtained with an Axon Instruments 200 patch-clamp amplifier, controlled and monitored with a PC running pCLAMP (ver. 6.0) with a 125-kHz interface (Axon Instruments, Foster City, CA). Electrode resistances were typically 2–4 MΩ in the bath. After seal rupture, series resistance (4–10 MΩ) was compensated (70–90%) and periodically monitored. Recordings were made only from large neurons (>14 pf) that had short (<75 μm) proximal dendrites; the identity of these neurons as cholinergic interneurons was verified with scRT-PCR (see Single-neuron RT-PCR). The adequacy of voltage control was assessed after compensation by examining tail currents generated by strong depolarization. Incomplete or discontinuous decay back to the baseline was taken as evidence of poor space clamp, and the cell was discarded.

**Cell-attached patch recording**

Recordings were obtained with an internal solution consisting of (in mM) 110 BaCl₂, 10 HEPES (pH 7.4 with TEA hydroxide). Electrodes were made as for whole cell recordings except that they were coated with silicone elastomer (Sylgard). After seal formation, the transmembrane potential was nominally zeroed by bathing the cell in an isotonic K⁺ solution containing (in mM) 140 K Gluconate, 1 MgCl₂, 5 ethylene glycol-bis-(β-aminoethyl ether)-N,N',N' ‑tetraacetic acid (EGTA), 10 HEPES, and 5 glucose (pH 7.4 with KOH, 300–305 mOsml). Transmembrane currents were evoked by stepping the electrode to −20 mV from a holding potential of +80 mV.

**Pharmacological methods**

Receptor ligands and second-messenger reagents were made up as concentrated stocks in water or dimethyl sulfoxide and stored at −80°C. Adenosine receptor ligands 2-chloro-N6-cyclopentyladenosine (CPA), CGS21680 hydrochloride (CGS), 8-cyclopentyl-1,3-dimethylxanthine (CPT), adenosine, and the muscarinic receptor agonist oxotremorine methiodide (oxo-M) were obtained from RBI (Natick, MA) or Tocris (Northpoint, UK). Phorbol-12-myristate-13-acetate (PMA) and 4a-phorbol were obtained from Sigma. The calcium channel blocker ω-conotoxin GVIA (ω-CgTx) was obtained from Peninsula Labs (Belmont, CA) or Calbiochem (San Diego, CA). Aliquots were thawed and diluted the day of use. Final dilutions were made in external media containing 0.01–0.1% cytochrome C when using ω-CgTx. The involvement of GIₒ proteins was studied using N-ethylmaleimide (NEM; Sigma).

Solutions were applied with a gravity-fed “sewer pipe” system. The application capillary (~150 μm ID) was positioned a few hundred micrometers from the cell under study. Solution changes were effected by altering the position of the array with a DC drive system controlled by a microprocessor-based controller (Newport-Klinger, Irvine, CA). Solution changes were complete within <1 s.

**Statistical methods**

Data were analyzed with AxoGraph (Axon Instruments, ver. 2.0) and SYSTAT (SPSS, Chicago, IL). Box plots were used for graphic presentation of the data because of small sample sizes (Tukey 1977). The box plot represents the distribution as a box with the median as a central line and the hinges as the edges of the box (the hinges divide the upper and lower halves of the distributions in half). The inner fences (shown as a line originating from the edges of the box) run to
the limits of the distribution excluding outliers (defined as points that are >1.5 times the interquartile range beyond the interquartiles); outliers are shown as asterisks or circles.

**Single-neuron RT-PCR**

As we have reported previously (Song and Surmeier 1996; Song et al. 1998), after recording, cells were lifted up into a stream of control solution and aspirated into the patch electrode by negative pressure. Electroporation, at ~5 μl of sterile recording solution (see above). Some cells were harvested without recording, with electrodes filled with diethyl pyrocarbonate (DEPC)-treated water. The capillary glass used for making electrodes was autoclaved and heated to 150°C for 2 h. Sterile gloves were worn during the procedure to minimize RNase contamination. After aspiration, the electrode was broken and contents ejected into a 0.5-mL Eppendorf tube containing 5 μl DEPC-treated water, 0.5 μl RNAsin (28,000 U/ml), and 0.5 μl diithiothreitol (DTT; 0.1 M). One microliter oligo(dT) (0.5 μg/μl) was added before the mixture was heated to 70°C for 10 min and incubated on ice for 1 min. Single strand cDNA was synthesized from the cellular mRNA by adding SuperScript II RT (1 μl, 200 U/μl), buffer (4 μl, 5 × First Strand Buffer: 250 mM Tris-HCl, 375 mM KCl, 15 mM MgCl2), RNasin (0.5 μl), DTT (1.5 μl, 0.1 M), and mixed dNTPs (1 μl, 10 mM). The reaction mixture (20 μl) was transferred to a 42°C water bath and incubated for 50 min. The reaction was terminated by heating to 70°C for 1 min. The mixture was then placed on ice. The RNA strand in the RNA-DNA hybrid was then removed by adding 1 μl RNase H (2 U/μl) and incubating for 20 min at 37°C. All reagents except for RNasin (Promega, Madison, WI) were obtained from Life Technologies (GIBCO BRL, Grand Island, NY).

The cDNA from the reverse transcription (RT) of RNA in single striatal neurons was subjected to polymerase chain reactions (PCR) to detect the expression of mRNAs coding for choline acetyltransferase (ChAT) and adenosine receptors. PCR amplification was carried out with a thermal cycler (MJ Research, Watertown, MA) with thin-walled plastic tubes. Reaction mixtures contained 2–2.5 mM MgCl2, 0.5 mM of each of the deoxynucleotide triphosphates, 0.8–1 μM primers, 2.5 U Taq DNA polymerase (Promega), 5 μl 10 × Buffer (Promega) and 4 μl of the cDNA template made from the single cell RT reaction. After a 5-min denaturing step to 95°C, a common thermal cycling program was executed for all primer sets: 94°C for 1 min, 56°C for 1 min, 72°C for 1.5 min. Forty-five PCR cycles were followed by a 10-min extension at 72°C. The ChAT mRNA (Brice et al. 1989) was identified using a pair of primers flanking a splicing site near the 3′ terminus of the coding region; the primers and conditions of this reaction have been published previously (Yan and Surmeier 1996). The primers for A1 receptor cDNA (Genbank accession No. M64299) (Maun et al. 1991) were 5′-AAC CTG AGT GTG GTA GAG CAA GAC-3′ (nucleotides 748–771) and 5′-A GTC CTC AGC TTT CTC TTC TGG G-3′ (nucleotides 1263–1285). This set yielded a product of 538 bp. The primers for A2a receptor (Genbank accession No. S47609) (Fink et al. 1992) were 5′-AG ATC ATC CGA ACCC AC CTC-3′ (nucleotides 961–983), and 5′-A CTC TGA AGA CCA TGA GGA AGCTC-3′ (nucleotides 1270–1293). This set yielded a product of 333 bp. The primers for A2b receptor (Genbank accession No. M91466) (Steinh et al. 1992) were 5′-AAT AAC AGC TGC TGC CCT GTG AAG-3′ (nucleotides 594–617), and 5′-GCT CAG CTC AAG GAA TGT CTA TCC-3′ (nucleotides 1077–1100). This set yielded a product of 507 bp. The primers for A3 receptor (Genbank accession No. M91452) (Zhou et al. 1992) were 5′-ATG TCC TGT GTG CCT GTG CTC-3′ (nucleotides 577–600), and 5′-CTC TGT GAA GCC ACG ATT CTG CTC-3′ (nucleotides 949–972). This set yielded a product of 396 bp. PCR products were visualized after separating by electrophoresis in 1.5–2% agarose gels and staining with ethidium bromide. Representative products were sequenced and found to match published sequences. Serial dilution experiments were performed using twofold dilutions of half the total cellular cDNA derived from the RT reaction (Barnauskas et al. 1999; Song et al. 1998; Tkatch et al. 1998).

Procedures designed to minimize the chances of cross-contamination were followed to carry out the PCR reactions (e.g., Cinino et al. 1990). Negative controls for contamination from extraneous and genomic DNA were run for every batch of neurons. To ensure that genomic DNA did not contribute to the PCR products, neurons were aspirated and processed in the normal manner except that the reverse transcriptase was omitted. Contamination from extraneous sources was checked by replacing the cellular template with water. Both controls were consistently negative in these experiments.

**RESULTS**

**Striatal cholinergic interneurons express primarily A1 and A2a adenosine receptor mRNAs**

After acute-dissociation of striatal tissue, large, presumptive cholinergic interneurons can readily be distinguished from medium spiny projection neurons by size (Fig. 1A). Large cells with a few primary dendrites (often 2 or 3) and whole cell capacitance >14 pF were chosen for recording. RT-PCR analysis of these neurons revealed that all expressed detectable levels of ChAT mRNA (n = 28; Fig. 1B, right panel).

To determine how the expression of adenosine receptor subtypes was coordinated in cholinergic interneurons, RT-PCR experiments were performed. As a positive control for PCR primers and amplification protocol, whole striatal mRNA was screened for adenosine receptors. As shown in Fig. 1B (left panel), all four adenosine receptor mRNAs were detected in the striatum. The amplicons were of the predicted size and sequence, verifying the selectivity of the amplification. Next, single cholinergic interneurons were examined. To maximize preservation of mRNA, neurons were aspirated without recording. Of 28 ChAT interneurons profiled using one-fifth of the cellular cDNA as a template, 90% had detectable levels of A1 adenosine receptor mRNA (Fig. 1B). On the other hand, A2a adenosine receptor mRNA was detected in only 27% of the interneurons tested (n = 33) using a similar protocol. To determine whether this reflected low transcript detectability or differential expression, serial dilution experiments were performed (Song et al. 1998; Tkatch et al. 1998). The distribution of detection thresholds for A2a receptor mRNA in a sample of 20 cholinergic interneurons is shown in Fig. 1C. The progressive increase in detection probability with increasing cDNA fraction suggests A2a mRNA was present in low abundance. Using a simple detection protocol with one-fifth of the total cellular cDNA, A2b adenosine receptor mRNA was found in an even smaller subset of identified interneurons (3/13). A3 receptor mRNA was not detected. Although quantitative analyses were not attempted for A3b mRNA, our interpretation of these results is that the low detection probability reflected low mRNA abundance (Song et al. 1998; Tkatch et al. 1998).

**Adenosine inhibits Ca2+ currents by activating A1 receptors**

Electrophysiological analysis was restricted to large (>14 pF) neurons that previous work had shown were cholinergic interneurons (Yan et al. 1997). RT-PCR analysis of a subset of neurons analyzed here (n = 12) confirmed their expression of ChAT. In these interneurons, adenosine rapidly and reversibly decreased Ba2+ currents evoked by depolarizing voltage steps (Fig. 2). The median reduction in peak Ba2+ current produced
ADENOSINE SIGNALING IN CHOLINERGIC INTERNEURONS

by 10 μM adenosine was 23% (n = 29). The reduction also was frequently accompanied by an alteration in current kinetics (Fig. 2A) and a small rightward shift in the current-voltage relationship (Fig. 2B). The application of the A1 receptor agonist CCPA (100 nM) also reversibly reduced the current. At the same concentration (100 nM), the A2a receptor agonist CGS-21680 did not detectably alter Ba2+ currents (n = 6, data not shown), suggesting that A2a receptors were not coupled to somatodendritic Ca2+ channels. This notion was further supported by experiments using the A1 receptor antagonist CPT. As shown in Fig. 3, CPT (1 μM) virtually eliminated the effects of adenosine (10 μM). Removing the antagonist restored the ability of adenosine to modulate currents. Similar results were seen in all cells tested (n = 8, P < 0.05, Mann-Whitney U test, see inset Fig. 3C). Dose-response experiments with A1- and A2a-selective agonists also supported this identification. As shown in Fig. 3D, the A1-selective agonist CCPA reduced Ba2+ currents with an IC50 of 45 nM. In contrast, the A2a-selective agonist CGS-21680 was much less effective, having an IC50 near 30 μM. These results suggest that adenosine inhibits Ca2+ channels by activating A1 receptors and that A2a receptors, although expressed, are not coupled to somatodendritic Ca2+ channels in this preparation.

NEM-sensitive G proteins mediate the A1 receptor action

To test for the involvement of G proteins, the impact of adenosine was examined with electrodes filled with 2 mM GDPβS (0 GTP). The nonhydrolysable GDPβS competes with endogenous GTP for the nucleotide binding site on Gα proteins, locking G proteins in an inactive state (Eckstein et al. 1979). Intracellular replacement of GTP with GDPβS almost completely eliminated the response to adenosine, suggesting that G proteins are involved in this process. The median modulation in cells dialyzed with GDPβS was 7% of that in control cells recorded the same day (n = 5, P < 0.05, Mann-Whitney U test). Previous electrophysiological studies have found that A1 receptors are coupled to pertussis toxin (PTX)–sensitive G proteins (G_{i/o}-class) (Scholz and Miller 1991; Zhu and Ikeda 1993). To test whether the A1 receptor effects in cholinergic interneurons also involved PTX-sensitive G proteins, the sulfhydryl alkylating agent, N-ethylmaleimide (NEM), was used. NEM has been shown to disrupt coupling of PTX-sensitive G proteins to Ca2+ channels (Shapiro et al. 1994). Unlike PTX, NEM acts quickly, allowing a positive control to be taken for the same cell. As shown in Fig. 4, adenosine (10 μM) and CCPA (100 nM) both reduced peak currents. A brief (2 min) application of NEM (50 μM) reduced the responses to both adenosine (n = 5) and CCPA (n = 3). Figure 4D shows the box plots of the percent modulation by adenosine and CCPA before and after the application of NEM in five experiments (CCPA was tested in 3 cells). NEM reduced both the adenosine and the CCPA modulation to ~20% of the control value, suggesting that the A1 receptor modulation was mediated by G_{i/o}-class G proteins.

A1 receptors inhibit N-type Ca2+ currents

Previous studies in several types of neuron have shown that A1 adenosine receptors inhibit N-type Ca2+ channels (Gross et al. 1989; Mynlieff and Beam 1994; Umemiya and Berger 1994; Zhu and Ikeda 1993). Striatal cholinergic interneurons have been shown to express five pharmacologically distinct types of Ca2+ channels, including N-type (Yan and Surmeier 2000). To determine whether A1 receptors in cholinergic interneurons also target N-type channels, the ability of α-CgTx GVIA to occlude the modulation was tested. As shown in Fig. 5, the application of α-CgTx GVIA (1 μM) eliminated the modulation of whole cell Ba2+ currents by adenosine. Shown in Fig. 5A is a time course from one of these experiments where peak current evoked by a voltage step to 0 mV is plotted as a function of time. Representative current traces are shown...
in Fig. 5B. Initially, the application of adenosine reduced peak currents. As expected, the application of ω-CgTx dramatically reduced peak currents. Subsequently, adenosine had little or no effect. A box plot summarizing the modulation by adenosine after the block of N-type channels (n = 4) is shown in Fig. 5A, inset. On average, >80% of the current reduction produced by adenosine was of N-type.

**A1 receptor modulation is membrane-delimited**

In other cells, A1 receptor activation of G i proteins leads to a reduction in cytosolic cAMP levels by inhibiting adenylyl cyclase (Linden 1991; Zink et al. 1995). However, previous work has failed to reveal any effect of cytosolic cAMP on voltage-dependent Ca2+ channels in cholinergic interneurons (Yan et al. 1997). On the other hand, G protein-coupled receptors can inhibit Ca2+ channels through a membrane-delimited pathway involving G protein βγ subunits (Herlitze et al. 1996; Ikeda 1996). One characteristic of this type of modulation is rapid onset kinetics. To test whether the A1 modulation of Ca2+ channels was fast enough to be consistent with this sort of mechanism, onset kinetics were measured at low and high agonist concentrations. In these protocols, a short (30 ms) depolarizing step to −20 mV was repeated once a second (faster rates led to N-type current inactivation), and CCPA was used instead of adenosine to minimize potential activation of other receptor subtypes. When applied at a high concentration (10 μM), CCPA rapidly reduced the current (Fig. 6A). The onset of the modulation was typically biexponential with a principal time constant near 1–2 s (Fig. 6B). At a lower agonist concentration (0.1 μM), the onset kinetics were slower (median, 3.2 s; n = 4). A box plot summary of the onset time constants at high (10 μM) and low (0.1 μM) agonist concentrations in four experiments is shown in Fig. 6B (inset). These onset kinetics are close to the range of those reported for membrane delimited signaling pathways in other cells (Hille 1994).

A more direct test of a membrane-delimited pathway is to bath apply agonist when recording in the cell-attached configuration of the cell membrane.
Adenosine inhibition of Ca\(^{2+}\) channels has been shown to be disrupted by activation of PKC in rat cortical, hippocampal, as well as sensory neurons (Swartz 1993). Muscarinic modulation of Ca\(^{2+}\) channels in cholinergic interneurons is disrupted by activation of PKC (Yan et al. 1997). To test whether the A\(_1\) receptor modulation possesses a similar sensitivity, PKC was activated by bath application of PMA. Before PMA treatment, both adenosine and oxo-M reduced evoked Ba\(^{2+}\) currents (Fig. 8, A and B). After PMA (500 nM) exposure, adenosine had substantially less of an impact on currents, as did oxo-M (Fig. 8, A and D). PMA appeared to specifically disrupt a component accompanying kinetic alteration (cf., Fig. 8, B and C). After PMA treatment, the effects of both adenosine and oxo-M were reduced to ~30% of control modulation. A box plot summarizing the results from this and three other experiments is shown in Fig. 8D. Application of the inactive phorbol analogue, 4a-ephorphol (500 nM), was without effect on both adenosine and oxo-M modulation (n = 6, Fig. 8D), arguing that the PMA effects were mediated by PKC activation.

The ability of PKC to disrupt both the A\(_1\) receptor and m2 receptor modulation suggests that they share common signaling elements. If this were the case, coactivation of the receptors should result in a subadditive modulation. To test this hypothesis, adenosine and oxo-M were co-applied. As shown in Fig. 9, in the presence of oxo-M, adenosine had little effect on depolarization-evoked Ba\(^{2+}\) currents. The median adenosine modulation in the presence of oxo-M was around 10% of the control modulation (see Fig. 9A, inset).

**DISCUSSION**

Cholinergic interneurons express A\(_1\) adenosine receptor mRNA

Large cholinergic interneurons could readily be visualized in the dissociated preparation and subsequently identified by RT-
PCR detection of ChAT mRNA. Ninety percent (25/28) of ChAT neurons had detectable levels of mRNA for the A1 adenosine receptor using one-fifth of the total cellular cDNA in the detection reaction. This percentage undoubtedly would have risen to near 100% had a larger fraction of the total cellular cDNA been used in the detection reaction. The inference that A1 adenosine receptor expression was ubiquitous is consistent with ability of adenosine to inhibit Ca2+ currents in every cell tested. In addition to A1 adenosine receptor mRNA, a substantial subset of interneurons (27%) had detectable levels of A2a receptor mRNA. Serial dilution experiments suggested that the abundance or detectability of this mRNA was low in cholinergic interneurons. No evidence was found for a subset of interneurons in which A2a mRNA abundance was high. In light of these results, the most parsimonious interpretation of our results is that A2a mRNA is present in all cholinergic interneurons, but at relatively low levels. Based on experiments

**FIG. 5.** Activation of A1 receptors reduces N-type Ca2+ currents. A: plot of peak current evoked by a step to 0 mV as a function of time and drug application. The modulation by adenosine was eliminated by block of N-type channels with ω-CgTx GVIA (CgTx, 1 μM). Inset: box plot summary of the percent control modulation by adenosine in the presence of CgTx (n = 4). B: representative current traces showing the modulation effect of adenosine before and after application of CgTx.

**FIG. 6.** A1 receptor modulation was rapid in onset. A: plot of peak current evoked by a step to −20 mV repeated at 1 Hz as a function of time and CCPA (10 μM) application. B: exponential fit of the onset of the modulation. Fitted line was determined by a least-squares algorithm; the fitted time constant is shown. Inset: box plot summary of onset time constants at high (10 μM; n = 5) and low concentration (0.1 μM; n = 4) of CCPA.

**FIG. 7.** Depolarizing prepulses attenuated the effects of A1 receptor activation on N-type currents. A: currents evoked by a step to −20 mV before and after adenosine (10 μM) application. B: currents in the same neuron evoked by the same step as in A, but preceded by a 30-ms step to +100 mV, in the presence and absence of adenosine. Note that the percent reduction was altered by the prepulse. Inset: box plot of the modulation produced by adenosine after a depolarizing prepulse (as a percentage of the modulation in the absence of a depolarizing prepulse) in a sample of 4 cells.
in medium spiny neurons where A2a receptor mRNA is readily detected (Song and Surmeier, unpublished observations), it is unlikely that the difficulty in detection was a consequence of inefficient reverse transcription or amplification. This interpretation is also consistent with previous in situ hybridization studies in which A1 receptor mRNA was more readily detected in cholinergic interneurons than A2a receptor mRNA (Dixon et al. 1996; Fink et al. 1992; Schiffmann et al. 1991; Svenningson et al. 1997). Although semiquantitative, single-cell studies were not attempted with A2b receptor mRNA, the most parsimonious interpretation of its infrequent detection is that it too is present in all cholinergic interneurons but at low levels.

**Activation of A1 receptors reduced N-type Ca2+ currents through a fast, membrane delimited, voltage-sensitive pathway**

Several lines of evidence suggest that the effects of adenosine on Ca2+ currents in cholinergic interneurons were mediated by A1 receptors. Beyond the virtual ubiquity of A1 receptor mRNA, the effects of adenosine were mimicked by nanomolar concentrations of the A1 receptor–selective agonist CCPA, but not by similar concentrations of the A2a-selective agonist CGS-21680. More detailed analysis of these agonists revealed nearly a thousand-fold difference in the IC50s for CCPA and CGS-21680 in modulating Ba2+ currents. Furthermore, the A1 receptor–selective antagonist CPT blocked the effects of adenosine. Third, the effects of adenosine were attenuated by brief exposure to NEM, which is known to disrupt signaling through Gβγ proteins (Shapiro et al. 1994). In contrast to A2a receptors, A1 receptors couple to intracellular signaling elements through Gβγ proteins (Linden 1991; Palmer and Stiles 1995).

Although A1 receptors are capable of inhibiting adenylyl cyclase (Linden 1991; Zink et al. 1995), their effects on Ca2+ currents were characteristic of a direct inhibition mediated by G protein βγ-subunits (Herlitze et al. 1996; Ikeda 1996). As in autonomic ganglion neurons, the reduction in evoked currents was largely occluded by the N-type channel-selective antagonist ω-CgTx GVIA. The modulation was rapid, having a time constant of a few seconds and was not seen in cell-attached patches when the agonist was applied outside the recorded patch. Both observations suggest a membrane-delimited signaling pathway. Last, as in other βγ subunit-mediated modulations of N-type channels, the inhibition of currents was accompanied by alteration in current kinetics that resembled changes seen in other cell types. This modulation was reversed by strong depolarization. Although a contribution by A2 adenosine receptors cannot be completely excluded, the broad outlines of this modulation are similar to those described in other cell types following activation of A1 adenosine receptors (Gross et al. 1989; Mynlieff and Beam 1994; Scholz and Miller 1991; Umemiya and Berger 1994; Zhu and Ikeda 1993).

**A1 receptor modulation was also PKC sensitive, much like that of the muscarinic autoreceptor**

The features of the A1 receptor modulation of Ca2+ currents are very similar to those of muscarinic m2/m4 receptors in cholinergic interneurons (Yan and Surmeier 1996). Activation of both m2/m4 and A1 receptors evoked a rapid, membrane delimited inhibition of N-type Ca2+ currents that was reversed by strong depolarization. In addition, both modulations were reversed by activation of PKC.
Like D₅ dopamine receptors, A₂ₐ adenosine receptors do not appear to couple to Ca²⁺ channels

Although cholinergic interneurons express low levels of A₂ₐ adenosine receptor mRNA, we found no evidence that these receptors (if present) couple to voltage-dependent Ca²⁺ channels. In other cell types, A₂ₐ receptors couple to Gₛ proteins, leading to the stimulation of adenyl cyclase and protein kinase A (PKA) (Brundege and Dunwiddie 1997). A₁ adenosine receptor activation has been reported to enhance P-type Ca²⁺ currents, presumably through a PKA-dependent mechanism (Mogul et al. 1993; Umemiya and Berger 1994). In striatal medium spiny neurons, stimulation of adenyl cyclase and PKA modulates voltage-dependent Ca²⁺ currents (Surmeier et al. 1995). It is possible that the enzyme digestion or the dissociation protocol employed in our experiments disrupted the ability of A₂ₐ receptors to couple to Ca²⁺ channels. These receptors may, for example, be present in distal dendrites that are lost during the dissociation. Nevertheless, the conclusion that A₂ₐ receptors expressed by cholinergic interneurons do not couple to somatic/proximal dendritic Ca²⁺ channels is in agreement with previous work using a similar preparation. In particular, activation of D₅ dopamine receptors or dialysis with cAMP analogues fails to modulate Ca²⁺ currents in cholinergic interneurons, in spite of the fact that these manipulations result in the modulation of GABA_A channels through a PKA-dependent mechanism (Yan and Surmeier 1997). The reasons for the apparent discrepancy are unclear. Cholinergic interneurons express Ca²⁺ channel α₁ subunits known to be targets of PKA (Yan et al. 1997). However, PKA may not be appropriately anchored to phosphorylate these channels in cholinergic interneurons (Gao et al. 1997; Klauck et al. 1996).

Inhibition of N-type Ca²⁺ currents provides a cellular mechanism for the effects of adenosine on ACh release

How A₁ receptor inhibition of N-type Ca²⁺ currents will affect synaptic integration and spike generation in cholinergic interneurons is unclear. N-type Ca²⁺ channels are found throughout the dendritic membrane of most types of neuron (Westenbroek et al. 1992). Reductions in dendritic Ca²⁺ currents could attenuate augmentation of excitatory synaptic events by voltage-dependent conductances (Bernander et al. 1994; Kim and Connors 1993). The A₁ modulation should also attenuate dendritic Ca²⁺ entry during back propagation of somatic spikes (Spruston et al. 1995).

The consequences of A₁ receptor modulation of N-type Ca²⁺ channels in synaptic terminals are more easily inferred. Ca²⁺ entry through N-type Ca²⁺ channels has been shown to be a major determinant of transmitter release in a variety of neurons (Dunlap et al. 1995). In cholinergic interneurons, A₁ receptor activation has been shown to reduce ACh release (Brown et al. 1990; Jin et al. 1993; Kirkpatrick and Richardson 1993). In all likelihood, this reduction in ACh release is dependent on A₁ receptor–mediated inhibition of N-type Ca²⁺ currents. The phenomenological similarities and shared signaling elements in the A₁ and m2/m4 muscarinic autoreceptor pathways reinforce this conclusion. Both receptors appear to be part of a negative feedback system; with terminal muscarinic m2/m4 receptors being stimulated by released ACh and termi-
nal A₁ receptors being stimulated by adenosine generated by the metabolism of co-released ATP (Richardson et al. 1987). The negative feedback regulation of ACh release through A₁ receptors should be particularly strong in the striosomes given this compartment’s prominent expression of 5′-nucleotidase (Schoen and Graybiel 1992). However, A₁ receptor inhibition of N-type Ca^{2+} channels also provides a mechanism for heterosynaptic inhibition of ACh release. Activity-dependent elevations in extracellular adenosine may result from the metabolism of transported cAMP or the direct release of adenosine (Bonci and Williams 1996; Brundege and Dunwiddie 1997; Harvey and Lacey 1997). ATP may also be released from corticostriatal glutamatergic terminals in an activity-dependent manner (Brundege and Dunwiddie 1997), providing yet another source of adenosine capable of inhibiting ACh release.

In contrast, our results do not provide an explanation for the ability of A_{2a} receptor agonists to increase ACh release (Brown et al. 1990; Kirkpatrick and Richardson 1993). Although cholinergic interneurons express A_{2a} receptor mRNA, agonists of these receptors had no obvious effect on Ca^{2+} channels linked to transmitter release. It is possible that A_{2a} receptor–mediated activation of PKA directly facilitates ACh release (Kondo and Marty 1997). Given the promise of A_{2a} receptor antagonists in treating Parkinson’s disease (Ferre et al. 1997; Kanda et al. 1998; Richardson et al. 1997), determining the functional impact of these receptors on cholinergic interneurons is an important task.

In summary, our results demonstrate that cholinergic interneurons express both A₁ and A₂ adenosine receptor mRNAs. Our results also demonstrate that A₁ adenosine receptor activation of Gi_{o/pro} proteins results in the inhibition of N-type Ca^{2+} channels through a rapid, membrane delimited signaling pathway that is sensitive to strong depolarization and protein kinase C. This signaling pathway provides a cellular mechanism for the A₁ receptor inhibition of striatal ACh release.

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