

# Role of the Afterhyperpolarization in Control of Discharge Properties of Septal Cholinergic Neurons In Vitro

NATALIA GORELOVA AND PETER B. REINER

*Kinsmen Laboratory of Neurological Research, Department of Psychiatry, University of British Columbia, Vancouver, British Columbia V6T 1Z3, Canada*

## SUMMARY AND CONCLUSIONS

1. The properties of the cholinergic neurons of the rat medial septum and nucleus of the diagonal band of Broca (MS/DBB) were studied using whole cell patch-clamp recordings in an in vitro slice preparation.

2. Both the transmitter phenotype and the intrinsic membrane properties of 56 MS/DBB neurons were determined post hoc by visualizing intracellularly deposited biocytin with fluorescent avidin and endogenous choline acetyltransferase with immunofluorescence. Twenty seven of 28 MS/DBB neurons exhibiting both a prominent slow afterhyperpolarization (sAHP) following a single action potential and anomalous rectification were identified as cholinergic. The remaining 28 neurons exhibited other intrinsic membrane properties and none were choline acetyltransferase immunoreactive.

3. The sAHP in MS/DBB cholinergic neurons was blocked reversibly either by reducing extracellular calcium or addition of 100  $\mu$ M cadmium and irreversibly blocked by 30 nM apamin, suggesting that the sAHP is produced by an apamin-sensitive calcium-activated potassium conductance.

4. MS/DBB cholinergic neurons also exhibited a postspike depolarizing afterpotential (DAP) preceding the sAHP. Both the DAP and the sAHP were blocked when extracellular calcium was lowered as well as in the presence of 10–50  $\mu$ M NiCl<sub>2</sub>. Application of 500 nM  $\omega$ -conotoxin also reduced the sAHP, while leaving the DAP intact. These data suggest that both transient and high-threshold calcium conductances contribute to generation of the sAHP.

5. When depolarized, cholinergic neurons fired slowly (2–4 Hz) and regularly with little evidence of spike frequency adaptation. When the sAHP was blocked with apamin, the instantaneous frequency of firing increased and the neuron now exhibited prominent spike frequency adaptation.

6. Serotonin (5-HT) reversibly suppressed the sAHP in MS/DBB cholinergic neurons and altered the firing pattern from slow regular discharge to one which exhibited modest spike frequency adaptation.

7. It was concluded that the sAHP limits the firing rate of MS/DBB cholinergic neurons and that physiologically relevant suppression of the sAHP by 5-HT may result in state-dependent changes in the discharge pattern of MS/DBB cholinergic neurons.

to different aspects of cognition and memory: cholinergic neurons in the nucleus basalis that project to the neocortex appear to be involved in attentional processes (Muir et al. 1994), while cholinergic neurons in the medial septum and vertical limb of the diagonal band of Broca (MS/DBB) that project to the hippocampus and cingulate cortex may be more important for the performance of conditional discrimination tasks (Marston et al. 1994). It has long been known that blockade of central muscarinic receptors with atropine blocks neocortical electroencephalogram desynchrony as well as theta activity in the hippocampus (Vanderwolf and Robinson 1981). Based on these and other observations, the basal forebrain cholinergic system has been implicated in control of arousal (Buzsaki et al. 1988; Semba 1991). Thus basal forebrain cholinergic neurons may be involved in both cognitive function and global shifts in cortical activity.

In contrast to the highly detailed understanding of the postsynaptic effects of acetylcholine in the forebrain (McCormick 1992; Nicoll et al. 1990), relatively little is known about the functional properties of identified forebrain cholinergic neurons. Several groups have investigated the intrinsic properties of presumed cholinergic neurons in brain slices, in acutely dissociated cells, and in primary cell culture (Allen et al. 1993; Alvarez de Toledo and López-Barneo 1988; Griffith 1988; Griffith and Matthews 1986; Griffith et al. 1994; Khateb et al. 1992; Markram and Segal 1990; Nakajima et al. 1985; Segal 1986). Of these studies, few have utilized choline acetyltransferase (ChAT) immunoreactivity to definitively identify physiologically characterized cholinergic neurons (Khateb et al. 1992; Markram and Segal 1990), while the remainder utilized either acetylcholinesterase histochemistry or cell size as an indicator of the transmitter phenotype of their recorded neurons. Given the importance of this area of the brain in models of cognitive function and arousal, it is imperative to develop a detailed understanding of the intrinsic membrane properties of known basal forebrain cholinergic neurons and their regulation by neurotransmitter agents. We have begun such a research effort and have carried out a series of experiments utilizing whole cell patch-clamp recording to study cholinergic MS/DBB neurons in rat brain slices. Here we present data indicating that the slow afterhyperpolarization (sAHP) has a powerful effect upon the discharge properties of cholinergic MS/DBB neurons and that serotonin (5-HT) can alter those discharge properties by inhibiting the sAHP.

## METHODS

### *Slice preparation*

Wistar rats of either sex and 18–28 days of age were anesthetized with halothane and decapitated. The whole brain was removed

## INTRODUCTION

Cholinergic neurons in the basal forebrain provide the major extrinsic cholinergic innervation of the hippocampus and cerebral cortex. These neurons are lost in the brains of patients with Alzheimer's disease (Coyle et al. 1983), and evidence exists implicating forebrain cholinergic mechanisms in cognitive function (Reiner and Fibiger 1995). Subsets of basal forebrain cholinergic neurons may contribute

rapidly and placed in ice-cold oxygenated artificial cerebrospinal fluid (ACSF). Coronal slices of 400- $\mu$ m thickness containing the MS/DBB complex were prepared on a vibratome and maintained in a custom-designed holding chamber at room temperature for  $\geq 1$  h before use. A single slice was transferred to the recording chamber, where it was continuously superfused with oxygenated ACSF at a flow rate of  $\sim 2$  ml/min.

### Electrophysiology

Patch pipettes were fabricated from borosilicate glass capillaries (1.5 mm OD) and exhibited resistances of 3–6 M $\Omega$  when filled with recording solutions. To reliably study the calcium-dependent sAHP of MS/DBB neurons (Griffith 1988) using whole cell patch-clamp recordings, we carried out a set of preliminary experiments in which we examined the sAHP from presumed cholinergic MS/DBB neurons while systematically manipulating the concentration of both calcium and ethylene glycol-bis( $\beta$ -aminoethyl ether)-*N,N,N',N'*-tetraacetic acid (EGTA) in the patch pipette. After considerable experimentation, we developed a solution that empirically appeared to buffer calcium sufficiently to allow cell survival but not so strongly that it prevented the appearance of the calcium dependent sAHP. This standard sAHP-recording internal solution contained (in mM) 120 K-gluconate, 25 NaCl, 0.025 CaCl<sub>2</sub>, 0.1 EGTA, 2 MgATP, and 10 *N*-hydroxyethylpiperazine-*N'*-2-ethanesulfonic acid; the pH of the solution was 7.25 when adjusted with KOH and yielded a final K<sup>+</sup> concentration of 140 mM. The free calcium concentration, calculated using the program MaxChelator (courtesy Chris Patton, Hopkins Marine Station) was 25 nM. For subsequent immunocytochemical study of the recorded cells, biocytin (0.2%) was added to internal solution. In some experiments, a more traditional whole cell patch pipette recording solution was employed, in which EGTA was increased to 11 mM and 1 mM CaCl<sub>2</sub> was added, yielding a calculated free calcium concentration of 10 nM. When a low chloride internal solution was employed, NaCl was replaced by equimolar Na-gluconate.

Whole cell patch-clamp recordings in brain slices were performed as previously described (Kamondi et al. 1992). Seal resistance was always  $>1$  G $\Omega$ . After rupture of the patch, access resistance was routinely  $<20$  M $\Omega$  or the cell was discarded. Signals were recorded with an Axoclamp-2A amplifier. Data were digitized at 49 kHz and stored on videotape for off-line analysis. For data acquisition and analysis, we used an Axolab interface and the PCLAMP suite of software (both from Axon Instruments). All experiments were performed at room temperature (22–25°C). Data are expressed as means  $\pm$  SD. Statistical comparisons were performed using Student's *t*-test, unless otherwise stated. Measures were accepted as statistically significant if  $P < 0.05$ .

### Solutions and drugs

The standard ACSF had the following composition (in mM): 126 NaCl, 2.5 KCl, 2.5 CaCl<sub>2</sub>, 1.2 MgCl<sub>2</sub>, 1.2 NaH<sub>2</sub>PO<sub>4</sub>, 26 NaHCO<sub>3</sub>, and 11 glucose. When saturated with 95% O<sub>2</sub>-5% CO<sub>2</sub>, the solution had a pH of 7.4. For low-calcium, high-magnesium containing solutions, CaCl<sub>2</sub> was reduced to 0.5 mM and MgCl<sub>2</sub> was increased to 10 mM. Low-sodium (27.2 mM) solution was made by equimolar substitution of choline chloride for NaCl. When studying voltage-activated calcium currents, 30 mM tetraethylammonium chloride (TEA) was added to the ACSF and NaCl reduced on an equimolar basis. In some experiments, tetrodotoxin (TTX, 300–500 nM) was added to block voltage-dependent sodium currents. Working solutions of CdCl<sub>2</sub>, NiCl<sub>2</sub>, 4-aminopyridine, apamin, and 5-HT were prepared just before use by diluting concentrated stock solutions in ACSF.

### Immunohistochemistry

After the conclusion of each electrophysiological experiment, the brain slices were fixed in 4% paraformaldehyde in 0.1 M phosphate buffer, pH 7.2 at 4°C for 1–2 h, washed in 0.1 M phosphate buffer, and stored overnight at 4°C in a cryoprotectant solution consisting of 30% sucrose in 0.1 M phosphate buffer. The following day, thin sections were cut at 35- $\mu$ m thickness on a cryostat, mounted on coated slides, and processed for both histochemical localization of biocytin and ChAT immunohistochemistry. Biocytin was visualized by incubation of the slides for 2 h at room temperature with fluorescein-5-isothiocyanate (FITC)-conjugated avidin (Vector Laboratories). After washing the slices in phosphate buffered saline, they were incubated for 48 h at 4°C with a monoclonal rat anti-ChAT antibody (Boehringer Mannheim). Next the sections were washed in phosphate buffered saline for 2 h at room temperature and incubated with Texas red-conjugated goat anti-rat antibody (Jackson Immuno Research Laboratories). Fluorescence microscopy, using appropriate filters for FITC and Texas red, was employed to identify the biocytin-filled and ChAT-immunoreactive neurons, respectively.

## RESULTS

### Identification of MS/DBB cholinergic neurons

Our data regarding the intrinsic membrane properties of neurons in the MS/DBB *in vitro* generally confirm the results of Griffith (1988). As in the guinea pig brain, neurons in the rat MS/DBB could be subdivided readily into three broad classes. The most obvious of these were those neurons that exhibited a prominent sAHP of 200- to 800-ms duration after a single action potential. A second class of neuron was observed that exhibited fast postspike afterhyperpolarizations (fAHP) of  $<100$ -ms duration. Finally, a third class of neuron was observed that exhibited burst-firing in response to depolarizing current pulses from membrane potentials negative to  $-60$  mV. Figure 1 illustrates the general properties of MS/DBB neurons as revealed by bridge mode recordings, and Table 1 provides quantitative data on some of these properties.

While these observations confirm earlier results, detailed examination of the responses of sAHP neurons to hyperpolarizing current pulses revealed two subpopulations (Fig. 1, A and B): one group of sAHP neurons exhibited only the rapid form of inward rectification known as anomalous rectification (sAHP-AR), whereas a second group of sAHP neurons (sAHP-IH) exhibited the time-dependent inward rectification, which manifests as a slowly developing depolarizing sag in the membrane potential, presumably due to the presence of a hyperpolarization-activated inward current (Kamondi and Reiner 1991). Of 129 recorded MS/DBB neurons, 72 were sAHP-AR, 14 were sAHP-IH, 20 were fAHP and 23 were burst-firing.

Next, we carried out experiments to determine which of these subgroups of neurons were cholinergic. Our approach involved combining intracellular deposition of biocytin with subsequent ChAT immunohistochemistry (Fig. 2). For inclusion in this experiment, cells were required to meet the following criteria: anatomic localization to the MS/DBB, well-characterized electrophysiological properties, successful biocytin labeling, and high-quality ChAT immunohistochemistry of the section containing the biocytin-labeled cell. Of 129 neurons tested, 56 MS/DBB neurons satisfied

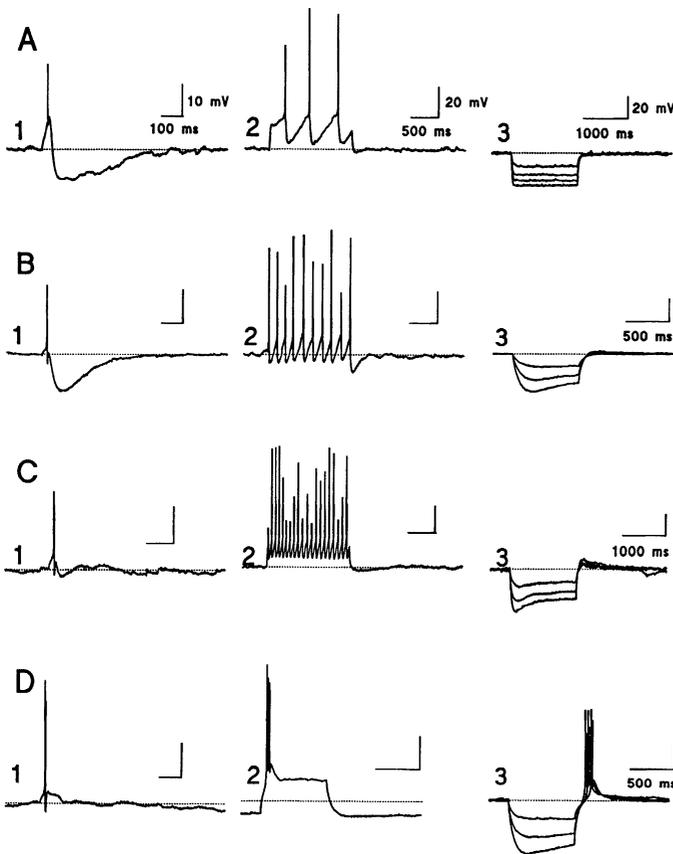


FIG. 1. Electrophysiological characteristics of medial septum and nucleus of the diagonal band of Broca (MS/DBB) neurons. A–D: slow after-hyperpolarization-anomalous rectification (sAHP-AR), sAHP-inward rectification (sAHP-IH), fast postspike AHP (fAHP), and burst-firing neurons, respectively.  $A_1$ – $D_1$ : response of MS/DBB neurons after a single action potential (height truncated) induced by short depolarizing current pulse (10 ms, 0.05–0.1 nA). Cells were held at  $-57$  mV.  $A_2$ – $D_2$ : responses of the same neurons to a prolonged depolarizing current pulse. Note that in  $D_2$ , the neuron is held at a slightly hyperpolarized membrane potential.  $A_3$ – $D_3$ : injection of hyperpolarizing current steps of 0.1, 0.2, 0.3, and 0.4 nA ( $A_3$ ) or 0.1, 0.2, and 0.3 nA ( $B_3$ – $D_3$ ) reveal different kinds of inward rectification—fast, time-independent for sAHP-AR neuron ( $A_3$ ), and time-dependent for others. Note that the burst-firing cell in  $D_3$  readily shows rebound burst discharge after the hyperpolarizing current step.

all of these criteria. Utilizing the electrophysiological classification scheme described above and shown in Fig. 1, this population of 56 biocytin-labeled neurons consisted of 28 sAHP-AR neurons, 9 sAHP-IH neurons, 8 fAHP neurons, and 11 burst-firing neurons. When these data were analyzed with respect to ChAT immunoreactivity, the results were remarkably consistent: 27/28 sAHP-AR cells stained positively for ChAT, while none of the other types of cells (including sAHP-IH neurons) were double labeled. Cholinergic MS/DBB neurons were silent at the resting membrane potential, which was  $-64.9 \pm 1.5$  mV ( $n = 27$ ).

These data unequivocally identify sAHP-AR neurons of the MS/DBB as cholinergic. The remainder of the paper describes a novel role of the sAHP in controlling the firing pattern of these cholinergic neurons. In every case, the properties we ascribe to cholinergic neurons were exhibited in identical fashion both by neurons definitively identified as cholinergic on the basis of double labeling as well as those identified only by their electrophysiological properties as sAHP-AR, and therefore we pooled the data.

### sAHP is an apamin-sensitive calcium-activated potassium conductance

The most remarkable feature of cholinergic MS/DBB neurons is the prominent sAHP that follows a single action potential. Because previous studies had suggested that the sAHP is mediated by activation of a calcium-activated potassium conductance (Griffith 1988), we developed a whole cell patch-clamp recording solution in which calcium was buffered at 25 nM (see METHODS), thereby allowing characterization of the sAHP under idealized recording conditions. Using such recording solutions, the sAHP at  $-57$  mV was  $375 \pm 95$  ms in duration and  $10.3 \pm 2.9$  mV in amplitude (Fig. 1,  $n = 27$ ).

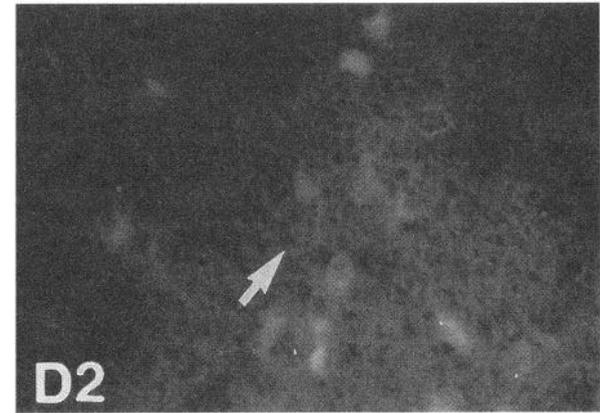
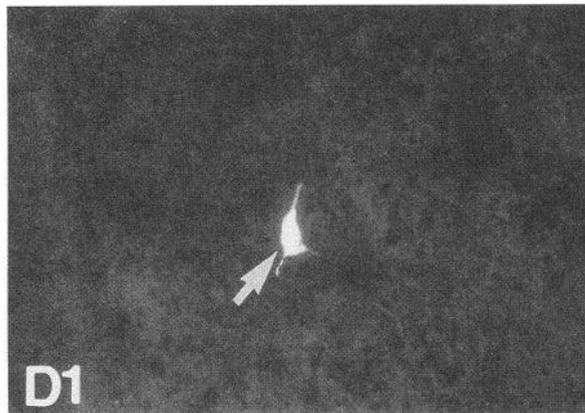
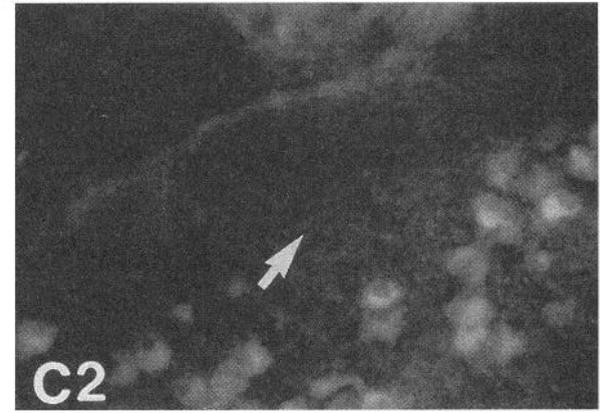
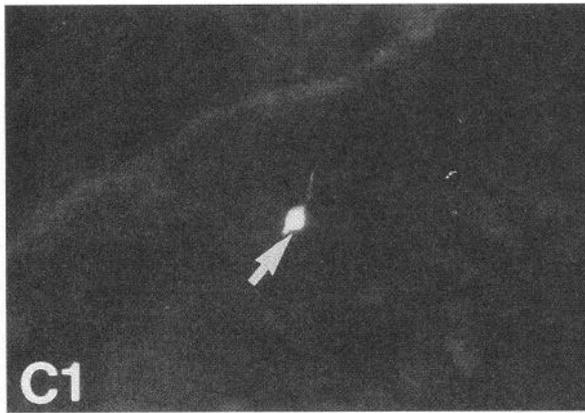
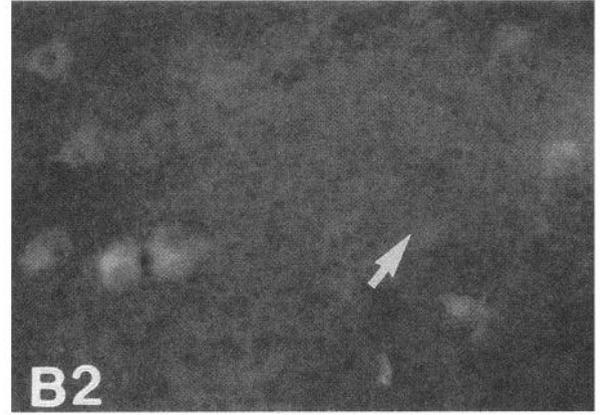
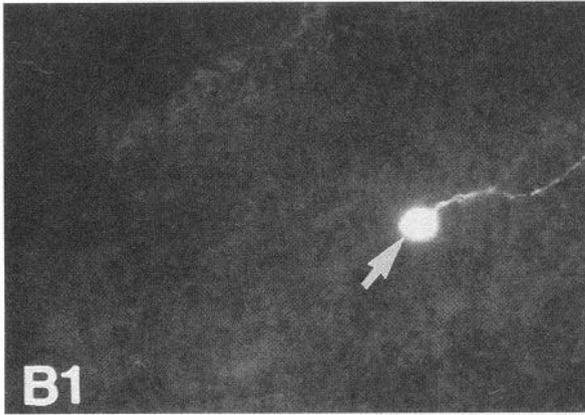
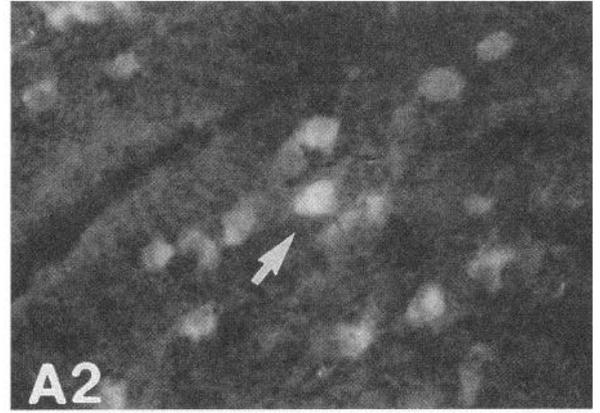
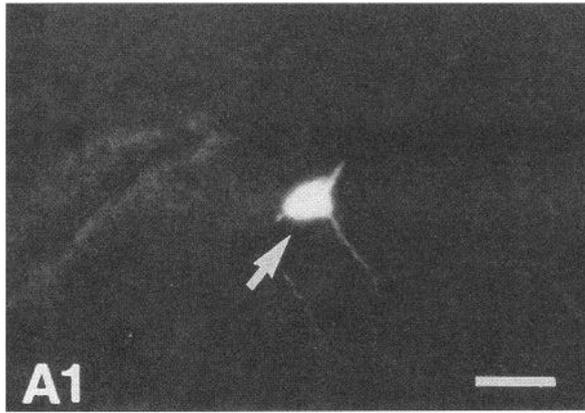
We next carried out experiments to investigate the calcium dependence of the sAHP. As previously described (Griffith 1988), when calcium in the ACSF was reduced to 0.5 mM and magnesium raised to 10 mM, the sAHP was reversibly reduced (Fig. 3A). Similar results were obtained when 100  $\mu$ M cadmium, a nonspecific calcium channel blocker, was added to the ACSF (Fig. 3B). We were also able to examine the effects of altering the concentration of calcium buffer in the intracellular medium upon the sAHP. During whole cell patch-clamp recording with solutions containing 10 mM EGTA and a calculated free calcium concentration of 10 nM, the duration of the sAHP after a single action potential at  $-57$  mV was  $152 \pm 17$  ms ( $n = 6$ ), which is significantly less ( $P < 0.05$ , Student's  $t$ -test) than that measured when the free calcium concentration was calculated to be 25 nM (above). In contrast to these effects on sAHP duration, the amplitude of the sAHP with 10 nM free calcium was not significantly different ( $9.1 \pm 1.4$  mV) from that measured in the presence of 25 nM free calcium (Fig. 3, D–F).

The data above are consistent with the hypothesis that the sAHP is produced by activation of a calcium activated potassium conductance. In most preparations, the sAHP is produced by activation of one or more small-conductance calcium-activated potassium channels. Some but not all of these channels are sensitive to blockade by the peptide toxin apamin (Blatz and Magleby 1986). The sAHP of cholinergic MS/DBB neurons was blocked irreversibly by apamin applied at 30–100 nM (Fig. 3C;  $n = 6$ ). Thus the sAHP is due to activation of an apamin-sensitive calcium-activated potassium conductance.

TABLE 1. Properties of subtypes of medial septal neurons

Cell Type	$n$	Resistance, M $\Omega$	AHP Amplitude, mV	AHP Duration, ms
sAHP-AR	51	$440 \pm 21$	$10.4 \pm 2.8$	$443 \pm 75$
sAHP-IH	11	$449 \pm 46$	$10.6 \pm 3.1$	$326 \pm 85$
fAHP	9	$352 \pm 45$	$3.4 \pm 1.3^*$	$31 \pm 8^*$
Burst firing	15	$596 \pm 71$		

Apparent input resistance was measured by injecting sufficient current to induce a hyperpolarization of  $<10$  mV at the resting potential. AHP amplitude represents the maximum excursion of the AHP measured at  $-57$  mV, and AHP duration represents the time that elapsed between the action potential and the return of the membrane potential to  $-57$  mV. sAHP, slow after hyperpolarization; sAHP-AR, sAHP-anomalous rectification; sAHP-IH, sAHP-inward rectification; fAHP, fast AHP. \* Significantly different from sAHP-AR,  $P < 0.001$ , Student's  $t$ -test.



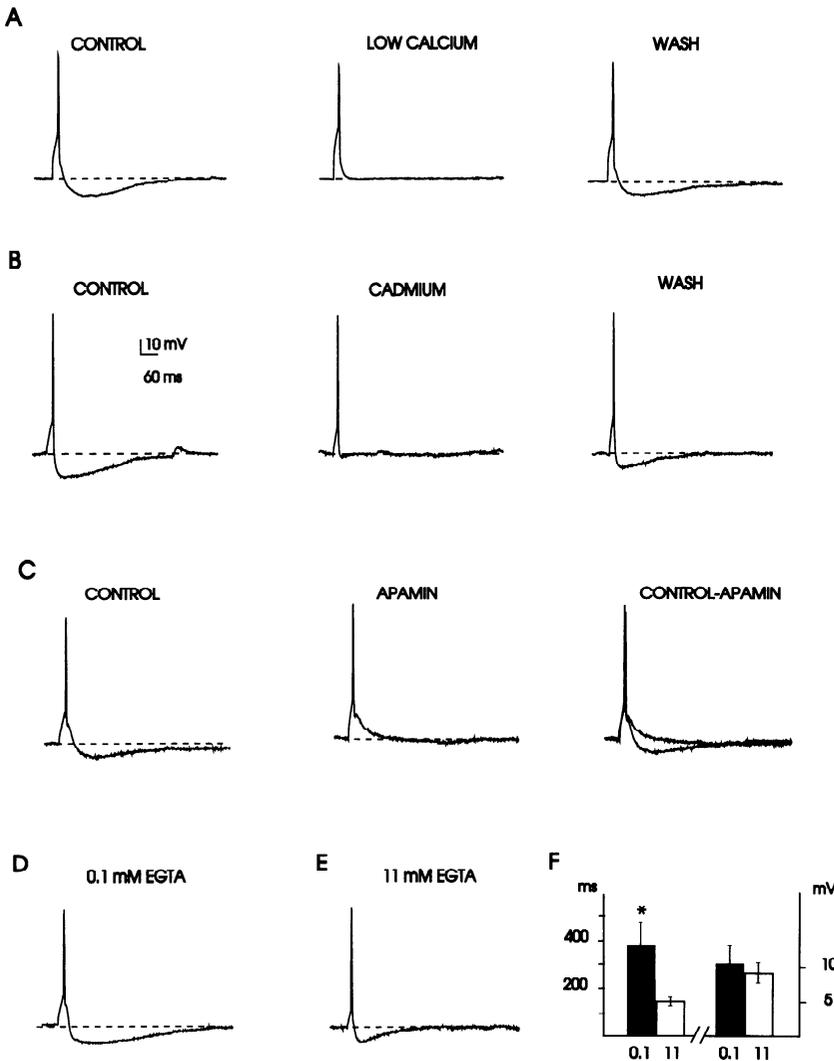


FIG. 3. Calcium dependence of the sAHP of MS/DBB cholinergic neurons. The sAHP after a single action potential (height truncated) is reversibly blocked in low-calcium (0.5 mM), high-magnesium (10 mM) ACSF (A), by addition of 100  $\mu$ M cadmium (B), or irreversibly by 30 nM apamin (C). Spikes were induced by short depolarizing current pulses (10 ms, 0.1 nA). D and E: representative recordings of sAHP observed when recording using patch pipette solutions containing 0.1 mM ethylene glycol-bis( $\beta$ -aminoethyl ether)- $N,N,N',N'$ -tetraacetic acid (EGTA, D) or 11 mM EGTA (E). Holding membrane potential is  $-57$  mV in all traces. F: summary bar graph showing the duration (left) and amplitude (right) of the sAHP recorded using 0.1 mM EGTA ( $n = 27$ ) and 11 mM EGTA ( $n = 6$ ) in the intracellular patch pipette solution. Note that duration but not the amplitude of the sAHP is significantly different under these treatment conditions.

*Both transient and high-threshold calcium conductances contribute to generation of the sAHP*

Preceding the sAHP, most cholinergic MS/DBB neurons exhibited a prominent postspike depolarizing afterpotential (DAP, Fig. 4A<sub>1</sub>). In cholinergic neurons in which a DAP was not evident, blockade of the sAHP with apamin usually revealed an underlying DAP (Fig. 4B). The DAP was not due to activation of voltage-sensitive sodium channels, as it persisted in the presence of 300 nM TTX (Fig. 4A<sub>2</sub>). Nor was it due to activation of a chloride conductance as it was observed in experiments in which the intracellular solution contained 40 mM Cl<sup>-</sup> as well as 0 Cl<sup>-</sup> in internal patch solution (data not shown); under the latter conditions, activation of a chloride conductance should result in a hyperpolarization. Changing the ACSF to one containing 0.5 mM Ca and 10 mM Mg blocked the DAP

(Fig. 4A<sub>3</sub>), suggesting that it was dependent upon an influx of calcium.

In many neurons, DAPs are produced by activation of a transient calcium conductance (Llinas 1988), which can be blocked somewhat selectively by micromolar concentrations of NiCl<sub>2</sub> (Fox et al. 1987). Application of 10–50  $\mu$ M NiCl<sub>2</sub> to the ACSF readily blocked the DAP (Fig. 4C<sub>3</sub>, and D). The sAHP was reduced by this manipulation as well, suggesting that calcium entry during the DAP contributes to generation of the sAHP. These data are consistent with the hypothesis that calcium entry during the DAP contributes to the sAHP.

We used pharmacological means to test the hypothesis that one or more high-threshold calcium conductances contribute to generation of the sAHP. Previous studies have shown that the peptide toxin omega-conotoxin inhibits high-threshold calcium conductances in basal forebrain cholinergic

FIG. 2. Identification of cholinergic and noncholinergic MS/DBB neurons. A<sub>1</sub>–D<sub>1</sub>: fluorescent photomicrographs of biocytin filled neurons revealed by fluorescein-5-isothiocyanate (FITC)-avidin. Physiological characteristics (see text) of the neurons are as follows: sAHP-AR (A<sub>1</sub>), sAHP-IH (B<sub>1</sub>), fAHP (C<sub>1</sub>), and burst-firing (D<sub>1</sub>). A<sub>2</sub>–D<sub>2</sub>: fluorescent photomicrograph of the same fields as on A<sub>1</sub>–D<sub>1</sub> (respectively) after choline acetyltransferase (ChAT)-immunohistochemistry using a Texas red-labeled secondary antibody. Only the sAHP-AR neuron (arrow in A<sub>2</sub>) stains positively for ChAT. Scale bar = 50  $\mu$ M.

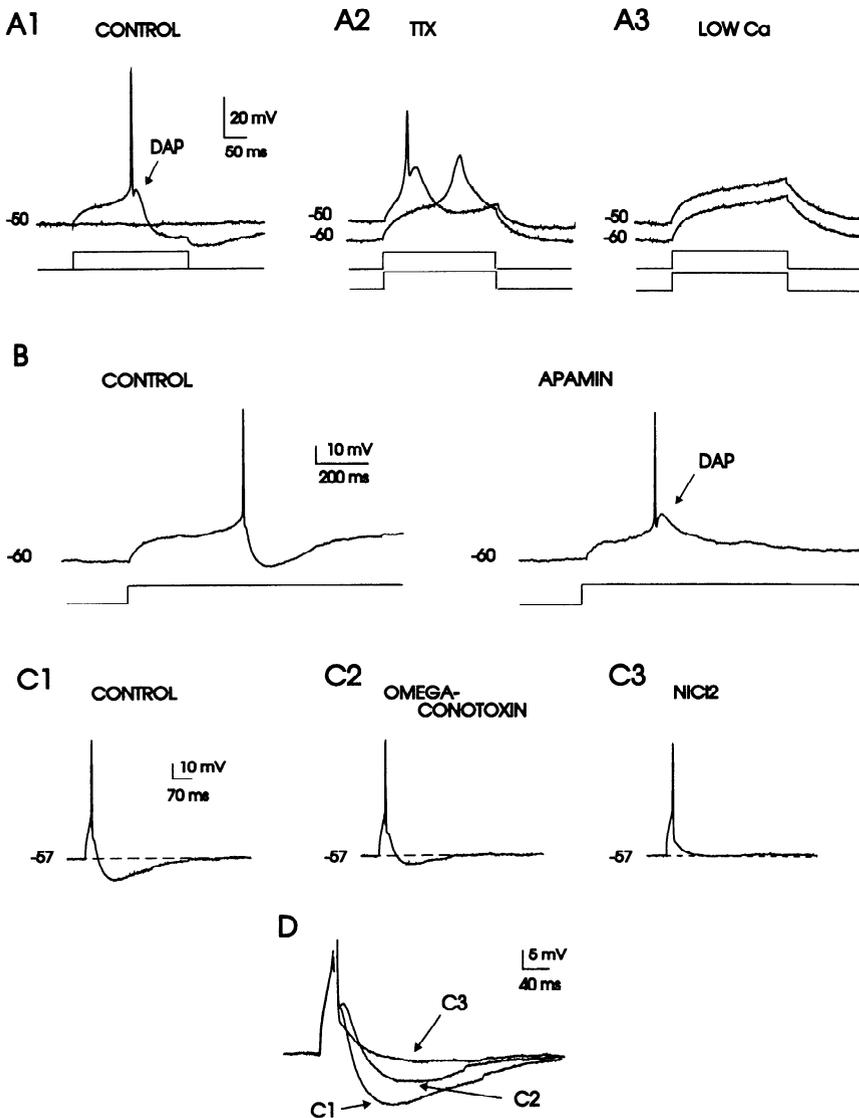


FIG. 4. Transient and high-threshold calcium conductances contribute to generation of the sAHP in cholinergic MS/DBB neurons. *A*<sub>1</sub>: a single action potential, induced by a 0.03 nA depolarizing step, is followed by both a depolarizing afterpotential (DAP) and sAHP. Holding potential is  $-50$  mV. *A*<sub>2</sub>: in the presence of 300 nM tetrodotoxin, the same depolarizing pulse induces both a large-amplitude, high-threshold calcium spike and a slower low-voltage-activated calcium spike with a time course similar to that of the DAP. Hyperpolarizing the membrane potential to  $-60$  mV permits selective activation of the low-voltage activated calcium spike. *A*<sub>3</sub>: reducing calcium to 0.5 mM and increasing magnesium to 10 mM blocks both calcium spikes. *B*: in a neuron that does not exhibit an obvious DAP under control conditions, application of apamin (30 nM) blocks the sAHP and reveals the presence of a DAP. The action potential was induced by application of a 0.04-nA depolarizing current step. *C*: application of  $\omega$ -conotoxin (500 nM, *C*<sub>2</sub>) reduced and nickel (10  $\mu$ M) eliminated the sAHP. *D*: expanded view of traces shown in *C* demonstrating that  $\omega$ -conotoxin did not eliminate the DAP while it is absent in the presence of nickel.

gic neurons (Allen et al. 1993; Griffith et al. 1994). Application of omega-conotoxin (500 nM) reduced, but did not eliminate, the sAHP ( $n = 5$ , Fig. 4*C*<sub>2</sub>, and *D*). We did not attempt to further define the high-threshold conductance using dihydropyridines, as these compounds also inhibit transient calcium conductances in basal forebrain cholinergic neurons (Allen et al. 1993). Taken together, these data demonstrate that both transient and high-threshold calcium conductances contribute to generation of the sAHP after a single action potential.

#### sAHP opposes spike frequency adaptation

The duration and magnitude of the sAHP that follows a single action potential suggests that it may be a potent regulator of the discharge properties of cholinergic MS/DBB neurons. Indeed, when using the standard sAHP-recording internal solution, the long duration of the sAHP apparently limited the frequency of firing, such that depolarizing current pulses of increasing intensity resulted in slow frequency firing of 2–4 Hz with little or no spike frequency adaptation

(Fig. 5*A*, 1–3). This regular pattern of discharge was altered readily when the sAHP was blocked by apamin. Under these conditions, the instantaneous rate of firing increased and, paradoxically, the neurons now exhibited strong spike frequency adaptation (Fig. 5*A*, 1'–3'). Thus specific blockade of the sAHP produced a profound change in the response properties of MS/DBB cholinergic neurons.

Experiments in which the concentration of intracellular calcium was altered support this conclusion as well. Although we were not able to record from the same neuron under conditions in which the intracellular calcium concentration was modified, spike frequency accommodation was encountered only rarely in neurons recorded with 0.1 mM EGTA in the patch pipette. On occasion, cholinergic septal neurons exhibited modest spike frequency accommodation (Fig. 9), but as in other studies of these neurons (Griffith 1988; Khateb et al. 1992; Markram and Segal 1990), this was very much the exception rather than the rule. When recordings were obtained using internal solutions containing 11 mM EGTA (buffering intracellular calcium to 10 nM), the duration of the sAHP was reduced (above), and neurons

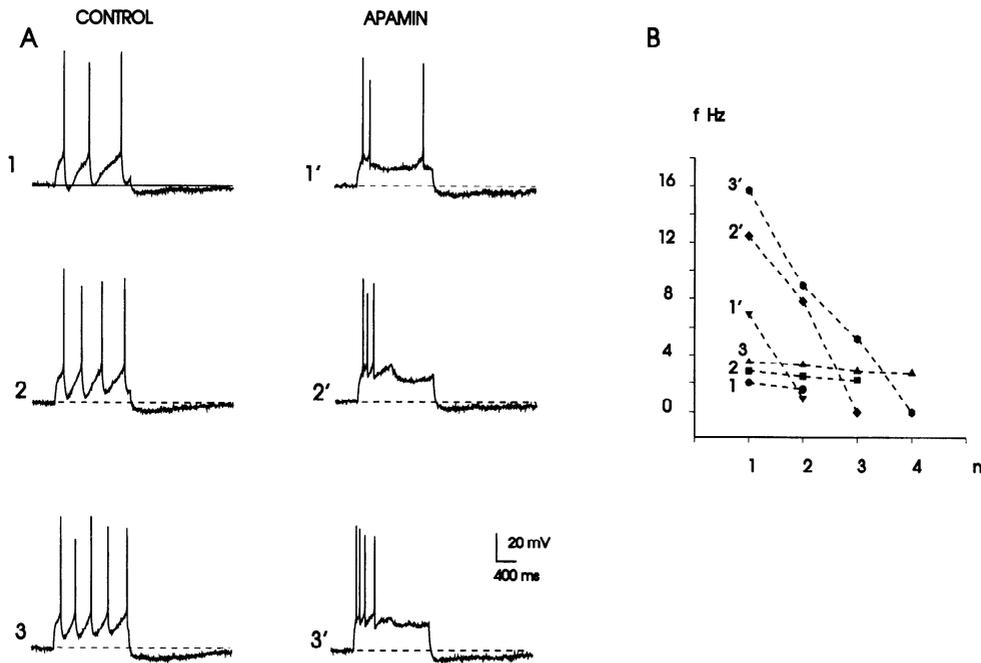


FIG. 5. Effects of apamin on discharge properties of cholinergic MS/DBB neurons.  $A_{1-3}$ : under control conditions, cholinergic neurons fire slowly and regularly in response to prolonged depolarizing current pulses of varying intensity (60, 90, and 120 pA).  $A_{1'-3}'$ : when 30 nM apamin is applied, the sAHP is blocked and the firing pattern changes to one exhibiting marked spike frequency adaptation. Note that AHP after the depolarizing step was not blocked by apamin.  $B$ : instantaneous frequency (f Hz) vs. spike number for the traces shown in  $A$  demonstrate absence of significant spike frequency accommodation under control conditions, and its emergence in the presence of apamin.

always exhibited spike frequency accommodation (Fig. 6). These data are in keeping with the hypothesis that the sAHP plays an important role in controlling the patterning of cholinergic MS/DBB neuronal activity.

In hippocampal neurons, spike frequency adaptation has been well studied and is to a large degree produced by activation of an apamin-insensitive calcium-activated potassium conductance (Lancaster and Adams 1986; Madison and Nicoll 1984; Storm 1989). To test the hypothesis that a similar

mechanism might be at play in MS/DBB cholinergic neurons, we examined their response properties following generalized blockade of calcium channels with cadmium. As expected, the sAHP was blocked reversibly by 100  $\mu$ M CdCl<sub>2</sub>, and the instantaneous firing frequency in response to depolarizing current pulses was dramatically increased (Fig. 7). However, under such conditions there was no spike frequency accommodation. Moreover, the sAHP that followed a train of action potentials under normal conditions was blocked;

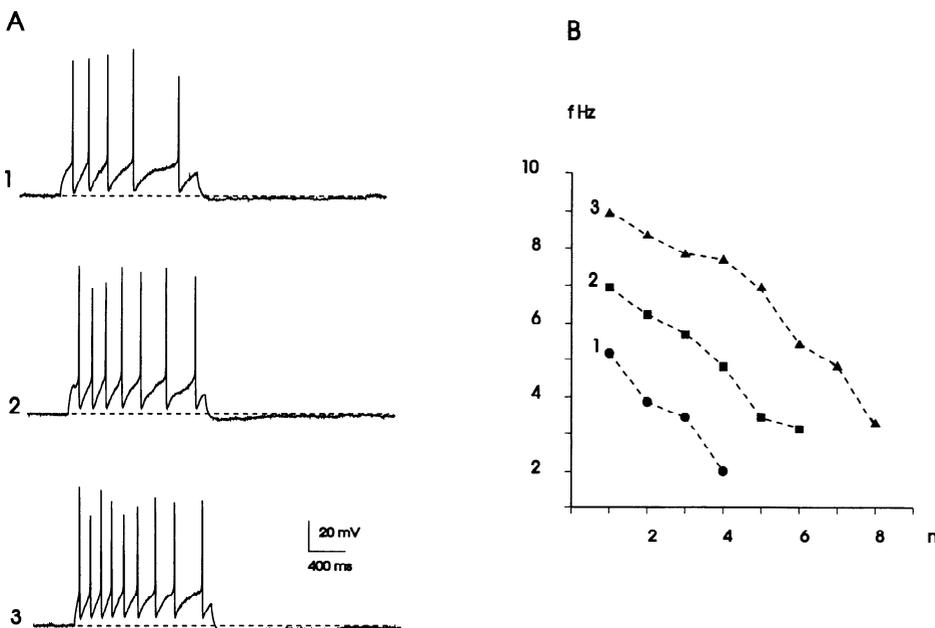


FIG. 6. Effects of increased intracellular calcium buffering upon spike-frequency accommodation in cholinergic MS/DBB neurons.  $A$ : recordings obtained from a neuron using 11 mM EGTA in the intracellular patch pipette solution demonstrate spike frequency accommodation in response to depolarizing current steps at three different intensities (60, 90, and 120 pA).  $B$ : instantaneous frequency (f Hz) vs. spike number for the traces shown in  $A$  demonstrate spike frequency accommodation at all intensities of stimulation.

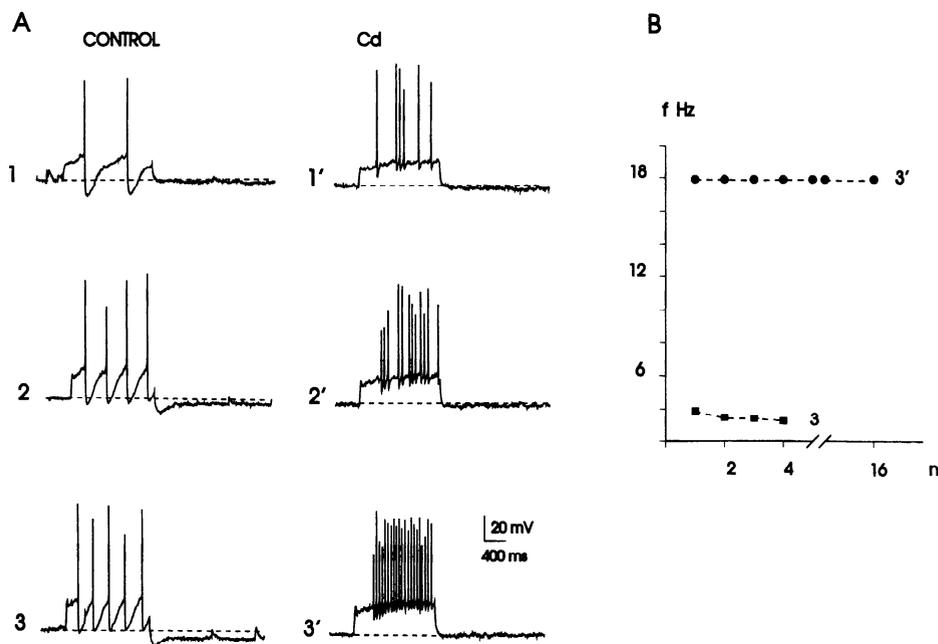


FIG. 7. Effects of cadmium on repetitive firing properties of cholinergic MS/DBB neurons. *A*: cadmium ( $100 \mu\text{M}$ ) blocks the sAHP and increases the number and frequency of action potentials evoked by intracellular injection of depolarizing current steps of 80, 120, and 160 pA. Note that cadmium also blocked the long-lasting AHP that follows the depolarizing current step. *B*: instantaneous frequency (f Hz) vs. spike number for traces  $A_3$  and  $A_3'$  demonstrate that cadmium increases firing rate but does not induce spike-frequency accommodation in these neurons.

it is notable that this form of sAHP was not blocked by apamin (Fig. 7A, 1'–3'). These data suggest but do not prove that in addition to the apamin-sensitive sAHP, there exists an apamin-insensitive calcium-activated potassium conductance in MS/DBB neurons that is at least partly responsible for spike frequency adaptation. Whatever the ionic basis of the phenomenon, it is only seen under conditions in which the sAHP that follows a single action potential is blocked. We next proceeded to determine whether this may happen under physiologically relevant conditions.

#### 5-HT blocks the sAHP and enhances spike frequency adaptation

Bath application of  $15\text{--}50 \mu\text{M}$  5-HT produced variable responses of cholinergic MS/DBB neurons in terms of changes in membrane potential. Of 26 cholinergic MS/DBB cells tested, 11 cells were depolarized by application of 5-HT, 12 hyperpolarized, and 3 showed a mixed depolarization-hyperpolarization sequence (Fig. 8). All responses persisted in the presence of 300 nM TTX as well as low-calcium- and high-magnesium-containing ACSF (data not shown), suggesting that the effects of 5-HT on membrane potential were direct.

In contrast to the variable effects of 5-HT on the membrane potential of MS/DBB cholinergic neurons, the effect of 5-HT on the sAHP was very consistent. In all neurons tested for effects on the sAHP ( $n = 10$ ), bath application of  $15\text{--}50 \mu\text{M}$  5-HT significantly reduced both the amplitude (from  $10.1 \pm 1.3$  to  $2.0 \pm 2.3$  mV,  $n = 10$ ) and the duration (from  $384 \pm 83$  to  $95 \pm 97$  ms,  $n = 10$ ) of postspike sAHP independent of the membrane potential response (Fig. 9A). As in case of apamin (but not so dramatically), reduction of the sAHP by 5-HT was accompanied by an increase in spike frequency adaptation (Fig. 9, B and C). Thus neurotransmitter agents can alter the discharge properties of cholinergic neurons by blocking the sAHP.

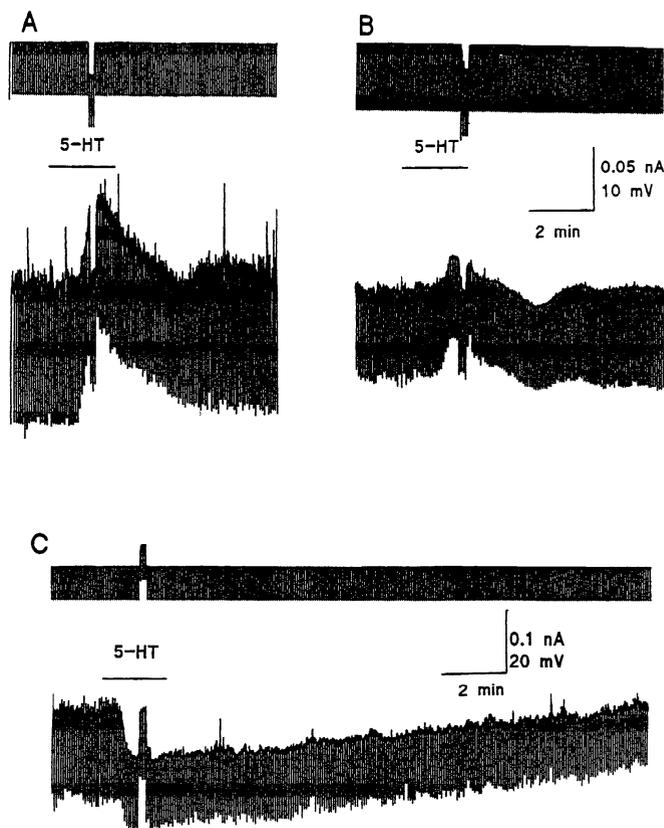


FIG. 8. Responses of the membrane potential of MS/DBB cholinergic neurons to serotonin 5-HT. *A–C*: whole cell patch-clamp recordings of the membrane potential of 3 different ChAT-positive MS/DBB neurons in response to bath application of  $30 \mu\text{M}$  5-HT. Negative going deflections on traces are electrotonic voltage transients produced by hyperpolarizing current steps (0.05 nA, 250 ms, 1-s interval) to monitor input resistance. During the period of application of 5-HT, the membrane potential was returned manually to the resting potential by current injection to monitor changes in input resistance. The holding potential is  $-58$  mV in all 3 cases. Periods of 5-HT application are indicated.

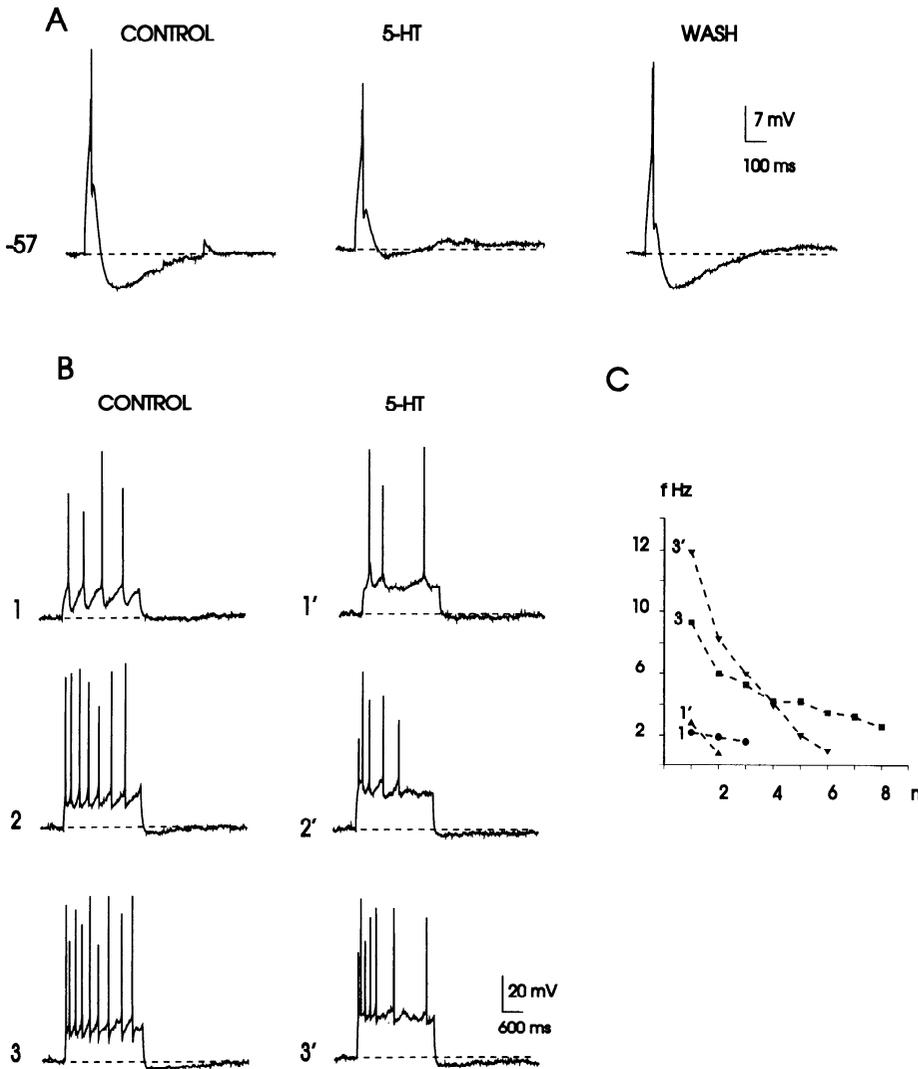


FIG. 9. 5-HT suppresses the sAHP and disrupts rhythmic firing of MS/DBB cholinergic neurons. *A*: traces showing the sAHP following a single action potential (height truncated) before and after application of  $30 \mu\text{M}$  5-HT. Spikes were induced by short depolarizing current pulses. Holding potential is  $-57 \text{ mV}$ . *B*: repetitive firing of the same as in *A* in response to prolonged (2 s) depolarizing current pulses before and during application of  $30 \mu\text{M}$  5-HT. Holding membrane potential is  $-60 \text{ mV}$ . *C*: instantaneous frequency (f Hz) vs. spike number for traces shown in *B* demonstrate that 5-HT enhances spike frequency accommodation.

## DISCUSSION

The major new findings of this study are that the sAHP that follows a single action potential *prevents* spike frequency accommodation in cholinergic MS/DBB neurons and that application of either apamin or serotonin, agents that block the sAHP, permits higher frequency and more irregular activity than is seen when the sAHP dominates. In addition, using a combination of intracellular labeling and ChAT immunohistochemistry, we have further delineated the intrinsic membrane properties of cholinergic neurons as revealed by whole cell patch-clamp recording in neonatal rat brain slices. We also have demonstrated the existence of a DAP in identified cholinergic MS/DBB neurons and have provided evidence that suggests that both transient and high-threshold calcium conductances contribute to generation of the sAHP.

### *Intrinsic properties of cholinergic MS/DBB neurons*

On the whole, our observations on the intrinsic membrane properties of cholinergic MS/DBB neurons are in accord with those obtained in the adult guinea pig (Griffith 1988; Khateb et al. 1992) and rat (Markram and Segal 1990)

basal forebrain. In particular, we were able to confirm that cholinergic MS/DBB neurons exhibit a prominent sAHP. However, the presence of the sAHP alone was insufficient to identify MS/DBB neurons as cholinergic. However, the association between neurons exhibiting sAHP and anomalous rectification was very strong, with 96% of such neurons being ChAT immunoreactive. In contrast, neurons exhibiting sAHP and  $I_h$  were never found to be cholinergic, and as in previous studies (Griffith 1988; Khateb et al. 1992; Markram and Segal 1990), neurons that did not exhibit any sAHP were also not ChAT immunoreactive. Given our sample size of 56 neurons that met the criteria of well-characterized electrophysiological recordings, successful biocytin labeling, and high-quality ChAT immunofluorescence, we feel confident in concluding that the sAHP-AR subtype of MS/DBB neurons represents an electrophysiological signature of cholinergic neurons in these brain slices.

We were able to show for the first time that identified cholinergic MS/DBB neurons exhibit DAPs. This finding is consistent with the observation that the more caudally located cholinergic nucleus basalis neurons exhibit both DAPs and transient calcium currents (Allen et al. 1993; Griffith et al. 1994; Khateb et al. 1992) and suggests that, like the

sAHP, this is a general property of basal forebrain cholinergic neurons.

It should be noted that although we were able to demonstrate the presence of a DAP, we were only occasionally able to provoke robust bursting activity in MS/DBB cholinergic neurons. Although the complement of ionic conductances exhibited by these neurons appears to be sufficient to demonstrate such behavior (Llinas 1988), it was rarely encountered. These results are in marked contrast to those obtained when recording from cholinergic neurons in the more caudally located nucleus basalis (Alonso et al. 1994; Khateb et al. 1992). It is unlikely that this is due to differences in temperature, as we were not able to observe bursts of action potentials crowning the DAP of MS/DBB cholinergic neurons even at 33°C (unpublished observations). It is also unlikely that this is due to a species difference, as guinea pig MS/DBB neurons exhibiting the sAHP also do not fire in bursts (Griffith 1988; Griffith and Matthews 1986). Finally, this does not appear to be developmentally regulated, as adult rat cholinergic MS/DBB neurons also rarely exhibit burst firing (Markram and Segal 1990). The lack of prominent burst firing behavior of cholinergic MS/DBB neurons may reflect a substantial functional difference between subsets of basal forebrain cholinergic neurons (Reiner and Fibiger 1995). However, it is also possible that the intrinsic properties of cholinergic MS/DBB neurons (in particular the role of the DAP) might be modified by neurotransmitter agents and that their behavior *in vivo* might not parallel that observed in slices of rat brain.

These observations are particularly important in light of the long-standing observation that there exist within the medial septum neurons exhibiting rhythmic bursting activity that is synchronous with the hippocampal theta rhythm (Alonso et al. 1987; Apostol and Creutzfeldt 1974; Petsche et al. 1962; Stewart and Fox 1989). Because such cells may act as pacemakers for hippocampal theta, much effort has been directed toward identifying which of the myriad types of neurons in the medial septum may be the elusive rhythmically active cells (Stewart and Fox 1990). Although our recordings using neonatal slices of rat brain are not absolutely predictive, the most parsimonious interpretation of the data is that the cholinergic neurons of the MS/DBB are poor candidates for such a role. Such a conclusion is concordant with recent data indicating that hippocampal theta persists after selective lesion of medial septal cholinergic neurons (Lee et al. 1994). Indeed, we feel that it is worthy of mention that the unidentified MS/DBB neurons that readily exhibit burst discharges would be much more likely to behave in such a fashion *in vivo*, and this is an issue worthy of further detailed investigation.

#### *Role of the sAHP in patterning the discharge properties of cholinergic neurons*

The key finding of the present study is that the presence of the sAHP in MS/DBB cholinergic neurons diminishes the likelihood of high-frequency firing and imposes upon these neurons a relatively regular pattern of activity. When the sAHP is blocked, the frequency of firing of MS/DBB cholinergic neurons is no longer limited, and more complex patterns of discharge can ensue. These observations are con-

cordant with data obtained from other types of neurons (Viana et al. 1993) and have considerable implications for the functions of cholinergic neurons.

The sAHP was blocked readily both by manipulations that reduce calcium entry and by apamin. Previous studies indicated that the reversal potential of the sAHP varied with the predicted potassium equilibrium potential (Griffith 1988) and that brief increases in intracellular free calcium precede and accompany the sAHP (Tatsumi and Katayama 1994). Taken together with our observations, it seems safe to conclude that the sAHP is due to activation of an apamin-sensitive calcium-activated potassium conductance. Both the kinetics and pharmacology of the conductance suggest that it is due to activation of small-conductance (SK) calcium-activated potassium channels. One property that distinguishes apamin-sensitive SK channels from the large-conductance calcium-activated channels is their calcium sensitivity: SK channels are activated by 100–300 nM calcium, whereas the large-conductance channels often require micromolar calcium for activation (Blatz and Magleby 1987). Consistent with these findings, it was estimated that the calcium concentration required for activating the sAHP in acutely dissociated nucleus basalis neurons was ~100 nM (Tatsumi and Katayama 1994). We have presented evidence suggesting that the calcium that triggers the sAHP derives both from a transient calcium conductance that appears to underlie the DAP and an omega-conotoxin sensitive high-threshold calcium conductance, however, this issue still requires further biophysical rigor. In particular, as of yet, we cannot state with any certainty whether other calcium conductances also contribute to generation of the sAHP.

In addition to the sAHP that follows a single action potential, we also were able to demonstrate that cholinergic MS/DBB neurons exhibit an apamin-insensitive AHP that follows a train of action potentials. In other neurons, the term sAHP has been applied to such hyperpolarizing potentials (Lancaster and Adams 1986; Madison and Nicoll 1984; Schwandt et al. 1992; Storm 1989; Viana et al. 1993), where it enhances spike frequency accommodation. It is possible that the apamin-insensitive AHP after a train of action potentials in MS/DBB neurons serves the same role, although we did not examine the issue in detail.

From the standpoint of cholinergic neuronal function *in vivo*, the observation that 5-HT can inhibit the sAHP is of paramount importance. The variable membrane potential responses to 5-HT are perplexing, particularly in light of previous studies demonstrating that 5-HT hyperpolarizes identified cholinergic nucleus basalis neurons (Khateb et al. 1993). However, our data indicating that 5-HT blocks the sAHP would predict increased responsiveness of cholinergic neurons under conditions in which serotonergic tone is increased. Consistent with this, 5-HT increases acetylcholine release in the hippocampus as measured by intracerebral microdialysis (Nilsson et al. 1992) and electrically evoked release of [<sup>3</sup>H] choline from slices of basal forebrain (Simiscalchi et al. 1994). As with 5-HT, muscarinic receptor stimulation can also inhibit the sAHP of cholinergic neurons (Sim and Griffith 1991). However, this does not appear to be a general feature of aminergic neurotransmitters, as neither histamine nor noradrenaline blocked the sAHP (unpublished observations).

Transmitter regulation of the sAHP is particularly important as it serves as the key ionic conductance regulating the firing frequency of MS/DBB cholinergic neurons. As has been described on numerous occasions, the classical firing pattern of cholinergic MS/DBB neurons in response to a tonic depolarizing current pulse is a slow, regular firing pattern (Griffith 1988; Griffith and Matthews 1986; Segal 1986). This pattern was modified by blockade of the sAHP: high-frequency firing now was observed, and in addition, cholinergic neurons exhibited spike-frequency accommodation. Serotonin-containing neurons exhibit marked state-related discharge patterns with highest activity during waking (Jacobs and Azmitia 1992). Our data therefore would suggest that the influence of serotonin during waking upon cholinergic MS/DBB neurons would be to alter their discharge properties and promote high-frequency firing. Indeed, a population of MS/DBB neurons change their pattern of activity from nonbursting to rhythmically firing during arousing stimuli (Sweeney et al. 1992). Our data represents one plausible cellular mechanism for such state-dependent changes in MS/DBB cholinergic activity.

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Address reprint requests to P. B. Reiner.

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