Proc. Natl. Acad. Sci. USA
Vol. 82, pp. 6344–6348, September 1985
Neurobiology

Two types of muscarinic response to acetylcholine in mammalian cortical neurons

(cingulate/M current/cholinergic/pirenzepine)

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Communicated by Richard F. Thompson, May 22, 1985

ABSTRACT Applications of acetylcholine (AcCho) to pyramidal cells of guinea pig cingulate cortical slices maintained *in vitro* result in a short latency inhibition, followed by a prolonged increase in excitability. Cholinergic inhibition is mediated through the rapid excitation of interneurons that utilize the inhibitory neurotransmitter γ-aminobutyric acid (GABA). This rapid excitation of interneurons is associated with a membrane depolarization and a decrease in neuronal input resistance. In contrast, AcCho-induced excitation of pyramidal cells is due to a direct action that produces a voltage-dependent increase in input resistance. In the experiments reported here, we investigated the possibility that these two responses are mediated by different subclasses of cholinergic receptors. The inhibitory and slow excitatory responses of pyramidal neurons were blocked by muscarinic but not by nicotinic antagonists. Pirenzepine was more effective in blocking the AcCho-induced slow depolarization than in blocking the hyperpolarization of pyramidal neurons. The two responses also varied in their sensitivity to various cholinergic agonists, making it possible to selectively activate either. These data suggest that AcCho may produce two physiologically and pharmacologically distinct muscarinic responses on neocortical neurons: slowly developing voltage-dependent depolarizations associated with an increase in input resistance in pyramidal cells and short-latency depolarizations associated with a decrease in input resistance in presumed GABAergic interneurons.

Acetylcholine (AcCho) is a putative neurotransmitter that is present both in neurons intrinsic to the mammalian cerebral cortex and in others that project to the cortex from the basal forebrain (1, 2). These cholinergic neurons give rise to synaptic terminals, which contact pyramidal cell dendrites and nonpyramidal cell bodies (3, 4). Application of AcCho to neurons of the cerebral cortex *in vivo* causes both inhibition and slow excitation (5). AcCho-induced slow excitation of neurons in the hippocampus, olfactory cortex, and sympathetic ganglion is mainly due to a decrease in a voltage-dependent potassium current (M current) (6–11). Slow cholinergic excitation in the neocortex appears to be mediated through a similar mechanism, although the voltage dependency of this action has not been well studied (12, 13). Cholinergic inhibition in the vertebrate nervous system has been studied only in the hippocampus (6, 7, 14, 15) and the sympathetic and parasympathetic ganglia (16–19), where it results from either a direct postsynaptic action of AcCho that produces an increase in membrane conductance to K^+ ions (15–18) or through excitation of interneurons (6, 7, 14, 19). The mechanism of cholinergic inhibition within the cerebral cortex is not known.

The cerebral cortex contains nicotinic as well as several subtypes of muscarinic AcCho receptors (20–23). Previous reports suggest that cholinergic slow excitation is mediated by receptors possessing muscarinic characteristics, while cholinergic inhibition may be due to activation of receptors that have both nicotinic and muscarinic properties (5, 24). The recent characterization of receptor antagonists (e.g., pirenzepine) and agonists (e.g., pilocarpine) that are relatively specific for subtypes of muscarinic receptors (21, 22) raises the question of whether different types of cholinergic responses demonstrated physiologically within the central nervous system might be due to activation of different subclasses of muscarinic receptors, as appears to be the case in parts of the peripheral nervous system (25). In the experiments reported here, we investigated this possibility for cholinergic inhibition and slow excitation of pyramidal neurons in the mammalian neocortex.

METHODS

Male or female guinea pigs (200–300 g) were anesthetized with Nembutal (30 mg/kg) and cooled in an ice bath to a core body temperature of 25°C–30°C. Slices of anterior cingulate cortex (500 μm thick) were cut in the coronal plane with a vibratome and maintained *in vitro* at 36°C–37°C by using standard techniques (26–29). After slices were incubated for at least 2 hr, we obtained intracellular recordings from 253 layer II–III neurons using beveled microelectrodes filled with 4 M KOAc (125–175 MΩ). AcCho agonists (10 mM) were introduced into the slice near the recorded neuron by applying a 10- to 20-msec pressure pulse (30–40 psi; 1 psi = 6.895 $\times 10^3$ Pa) to the back of a two-barrel broken micropipette. The volume of each application was 10–20 pl. In pyramidal neurons, changes in input resistance were calculated from current–voltage relationships before and after AcCho administration by using an active bridge circuit. Dihydro-β-erythroidine was a gift from Merck Sharp & Dohme and McN-A-343, pirenzepine hydrochloride, and oxotremorine-M bromide were gifts from Donald Jenden. All other compounds were obtained from Sigma. The effects of antagonists were analyzed by comparing the responses of populations of neurons to AcCho in the same slices before and after at least 1 hr of exposure to the antagonist-containing medium, or by comparing the responses of single neurons to AcCho before and after local or bath applications of the antagonist. Nearly all (>90%) pyramidal neurons recorded in normal solution gave robust biphasic responses to AcCho if the application was within 75 μm of the recorded cell. Therefore, in the presence of antagonists, only AcCho applications that were also within 75 μm of the recorded neuron were analyzed. When cholinergic agonists were used, applications of AcCho were first used to find the depth in the slice where the best hyperpolarizing and slow depolarizing responses could be

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Abbreviations: AcCho, acetylcholine; GABA, γ-aminobutyric acid; DMP, 1,1-dimethyl-4-phenylpiperazinium iodide.

evoked. The cholinergic agonist was then applied to the same neurons from the second barrel of the dual-barrel micropipette. Applications of all agonists were also carried out with two independent micropipettes to rule out cross-contamination of the solutions in the dual-barrel pipette. Data obtained by the two methods were identical and therefore were combined.

Intracellular recordings from neurons in the cortical slice preparation have revealed the presence of two broad classes of neurons: regular-spiking and fast-spiking. Fast-spiking neurons are distinguished from regular-spiking neurons by action potential durations of <1.0 msec (at base), the ability to generate high frequency (>250 Hz) trains of action potentials, and the presence of prominent spike afterhyperpolarizations (27). Intracellular injections of the fluorescent dye Lucifer yellow CH (27, 28) revealed that the regular-spiking neurons are pyramidal in morphology, while fast-spiking neurons are aspiny or sparsely spiny interneurons and are similar or identical to those neurons that utilize the inhibitory neurotransmitter γ-aminobutyric acid (GABA). We use the terms pyramidal neuron and interneuron here to indicate these two classes of cells.

RESULTS

We have previously found that the actions of AcCho on presumed pyramidal and interneuron subtypes of cortical cells differ dramatically (30). These results will be published in detail elsewhere; however, pertinent portions of these data relevant to interpretation of the present pharmacological experiments are summarized here.

Applications of AcCho to presumed pyramidal neurons of the anterior cingulate, sensorimotor, and visual cortical regions at resting membrane potential (V_m, approximately −75 mV) cause a short latency inhibition associated with a decrease in input resistance (R_N) without significant fast or slow depolarizations (Fig. 1*A*, i). However, after this initial inhibition, the response to depolarizing current pulses is enhanced, without changes in resting V_m or the response of the cell to hyperpolarizing inputs (Fig. 1*A*, e). The latter result indicates that in these neurons, AcCho may affect a voltage-dependent current that is most active at depolarized levels. Indeed, applications of AcCho to presumed pyramidal neurons that are first depolarized to near firing threshold with intracellular current result in short latency (typically <50 msec) hyperpolarization, which often appear as a barrage of inhibitory postsynaptic potentials (Fig. 1*B*, i), followed by a longer latency, prolonged depolarization, and action potentials (Fig. 1*B*, e). Thus, the slow depolarizing response in pyramidal neurons is a voltage-dependent event that is small or does not occur at membrane potentials hyperpolarized to approximately −65 mV.

The initial inhibitory response appears to be mediated through a rapid excitation of inhibitory interneurons because it has a reversal potential similar to the hyperpolarizing response produced by GABA and is blocked by drugs that prevent GABAergic synaptic transmission (bicuculline, picrotoxin, tetrodotoxin, high Mn^{2+}, low Ca^{2+}) (30). Application of AcCho to physiologically identified presumed GABAergic interneurons results in short onset latency (typically <50 msec) excitation (Fig. 1*C*) associated with a large decrease in R_N (Fig. 1*D*). This response is increased or decreased in amplitude as V_m is increased or decreased, respectively (Fig. 1 *C* and *D*), and can be reversed during large current-imposed depolarizations. The duration of this excitation is similar to that of the AcCho-induced inhibitory response seen in isolation in pyramidal neurons when AcCho is applied at a distance (>100 μm) from the soma.

From these data, it appears that two different types of excitatory AcCho action are present throughout the cerebral cortex and, furthermore, that they are segregated on two classes of neurons. We sought to further differentiate these responses by their pharmacological characteristics. The initial inhibitory phase of pyramidal cell responses to AcCho was used as an index of interneuronal depolarization by this agent.

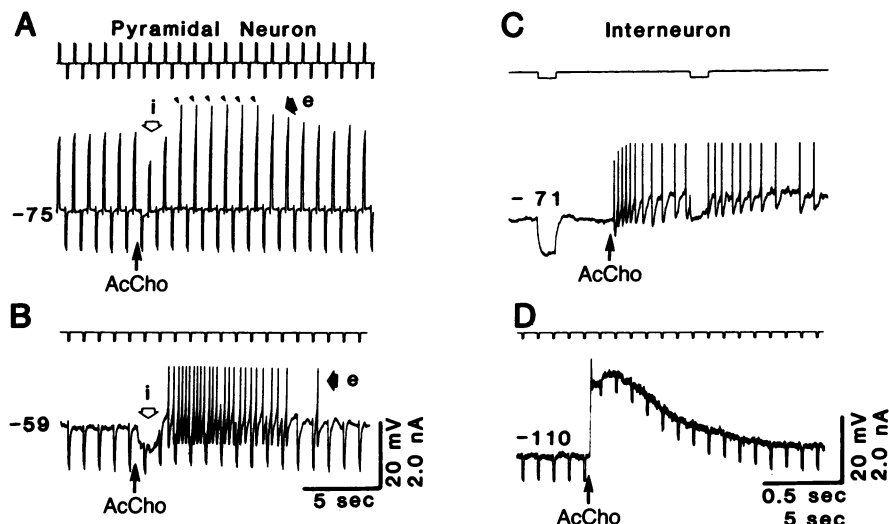

FIG. 1. The effects of AcCho on physiologically identified pyramidal cells and interneurons. (*A*) Application of AcCho to a typical pyramidal cell at resting membrane potential (V_m = −75 mV) initially caused a decrease in the response to the current pulses (i) followed by a selective potentiation of the depolarizing responses without affecting resting V_m or the response to the hyperpolarizing pulses (e). The potentiated depolarizing pulses reached firing threshold and evoked action potentials (downward arrowheads). (*B*) Application of AcCho to the neuron from *A* after depolarization to near firing threshold (−59 mV) caused inhibition at a short latency (i) and was followed by a slow depolarization and action potential generation (e). (*C*) Application of AcCho to a typical interneuron at resting V_m (−71 mV) caused robust excitation at short latency. (*D*) Application of AcCho to the interneuron from *C* after hyperpolarization to −110 mV evoked a large depolarization with a short onset latency. Comparison of responses to hyperpolarizing pulses during AcCho-induced depolarization with responses during equivalent direct depolarizations showed that AcCho elicited a substantial decrease in input resistance (not shown). The top trace in each set is the current monitor. In this and all subsequent figures, the intracellular current pulses are 120 msec in duration and were applied at 1 Hz. Action potential amplitudes are truncated. Time calibration is 0.5 sec for *C* and 5 sec for *A*, *B*, and *D*.

Bathing the slices in medium containing the muscarinic antagonist atropine sulfate salt (1 or 10 μM; n = 16) blocked all responses to AcCho without affecting action potentials or postsynaptic potentials, whereas 0.1 μM atropine was insufficient to block either the hyperpolarizing or slow depolarizing AcCho response (n = 6). Brief local surface application of scopolamine (10 μM) also completely blocked all actions of AcCho (Fig. 2A). Nicotinic antagonists hexamethonium bromide (10 μM; n = 3) or dihydro-β-erythroidine (10 or 100 μM; n = 10) did not block the typical biphasic AcCho response. These results indicate that the receptors mediating both the inhibitory and the slow excitatory responses of pyramidal neurons are muscarinic in nature (5). In agreement with this, applications of nicotinic agonists nicotine and 1,1-dimethyl-4-phenylpiperazinium iodide (DMP; n = 5) did not elicit the slow depolarizing response, while DL-muscarine was found to be a very potent agonist in activating this response (Table 1). However, nicotine, DMP, and DL-muscarine were all weakly effective in causing inhibitory responses in pyramidal neurons (Table 1; Fig. 2C). Interestingly, after applications of nicotine or DMP, further applications of AcCho caused the slow depolarization response only (i.e., the hyperpolarizing phase was blocked). Application of DMP to an interneuron mimicked the action of AcCho in that it caused a rapid, but weaker, excitatory response. These results indicate that, although the receptors mediating the rapid excitation of interneurons are largely muscarinic in nature, they may also be sensitive to some traditional nicotinic agents (24).

Pirenzepine is a muscarinic antagonist that has relative selectivity for a particular subclass of muscarinic binding sites (21, 22, 31). Bath applications of pirenzepine blocked the actions of AcCho in a dose-dependent manner. Neurons obtained from slices bathed in 1 μM pirenzepine responded to AcCho with both hyperpolarizing and slow depolarizing responses, although the latter were relatively weak (n = 4). Neurons impaled in slices bathed in 10 μM pirenzepine generated only hyperpolarizing responses to AcCho (n = 8); slow depolarizing responses were absent (n = 7) or very weak (n = 1) (Fig. 3A, post). Cells obtained in 50 μM pirenzepine either did not respond to AcCho, or they generated only small hyperpolarizing responses (n = 10). Therefore, pirenzepine, but not atropine, appears to differentiate between the muscarinic receptors mediating cholinergic inhibition and slow excitation of pyramidal neurons. To further test this possibility, we maintained intracellular recordings from single pyramidal neurons during wash-in and wash-out of 10 μM pirenzepine. Pirenzepine significantly diminished or abolished the slow depolarization (t = 8.5, df = 5, $P < 0.001$, n = 6; t test of difference scores) without affecting the inhibitory response (t = 0.1, NS, n = 6) (Fig. 3). The slow excitatory response recovered significantly during wash-out of the pirenzepine (t = 8.0, df = 2, $P < 0.05$) (Fig. 3). Therefore, pirenzepine preferentially antagonized the actions of AcCho at the sites mediating the slow depolarizing response.

We sought to further characterize the pharmacological profile of the muscarinic receptors mediating these two

Table 1. Comparison of efficacy of cholinergic agonists in generating inhibitory and slow excitatory responses in cortical pyramidal cells

Agonist	Inhibition	Slow excitation
Carbachol	++++	+++
AcCho	+++	+++
Methacholine	+++	+++
Propionylcholine	+++	++
Oxotremorine-M	++	++++
Oxotremorine	++	−
Muscarine	+	++++
Suberyldicholine	+	++++
McN-A-343	+	+
Nicotine	+	−
DMP	+	−
Pilocarpine	−	+/++

++++, More effective than AcCho; +++, equally effective; ++, less effective; +, weakly effective; −, inactive. Number of cells is indicated in text.

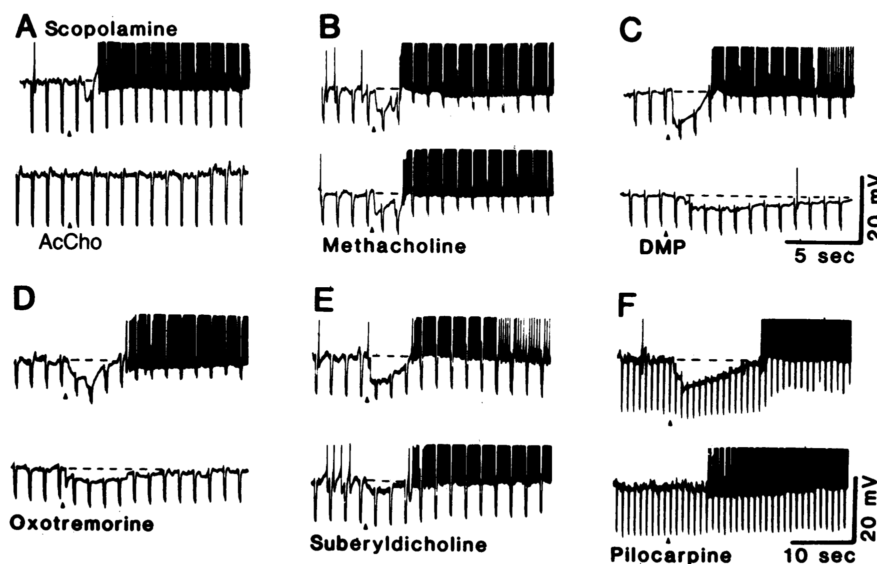

FIG. 2. Comparison of responses of various cholinergic agents with those to AcCho. In all sets, the top recording is the response to AcCho in normal solution. (*A*) Brief local application of the muscarinic antagonist scopolamine completely blocks all actions of AcCho (bottom trace, postscopolamine). (*B*) Application of the muscarinic agonist acetyl β-methacholine causes the typical biphasic response. (*C* and *D*) Application of the nicotinic agonist DMP and the cholinergic agonist oxotremorine both cause the hyperpolarizing response only. (*E*) Applications of the cholinergic agonist suberyldicholine caused relatively weak inhibition, followed by strong slow excitation. (*F*) Pilocarpine did not cause the inhibitory response, but it was capable of generating the slow excitatory response. Volume of application of pilocarpine in this cell was 4 times that of AcCho.

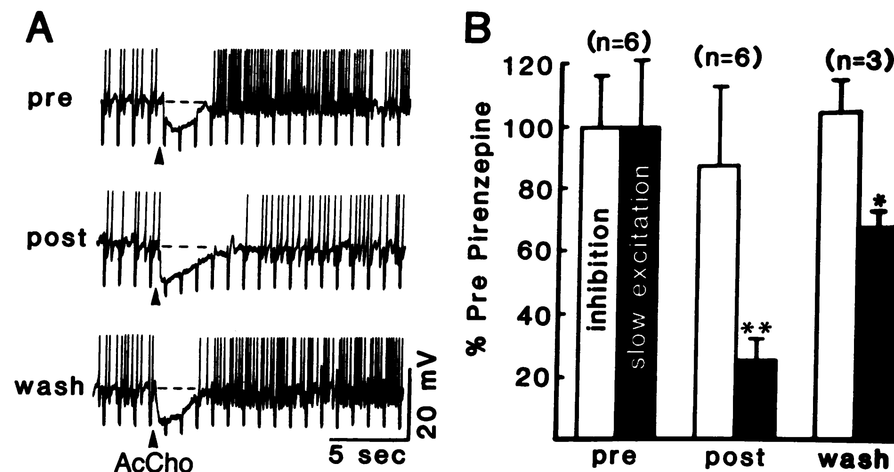

FIG. 3. Effects of 10 μM pirenzepine on the inhibitory and slow excitatory responses to AcCho. (A) Application of AcCho (upward arrowhead) to this neuron at V_m just above firing threshold evokes the typical inhibitory slow excitatory response (pre). After ≈1 hr of exposure to 10 μM pirenzepine, AcCho application to the same neuron causes the inhibitory response only (post). Washing out the pirenzepine for ≈1 hr partially reinstates the slow excitatory response (wash). (B) Average data for six neurons showing that 10 μM pirenzepine causes a significant (**, $P < 0.001$) depression of the slow excitatory response without affecting the amplitude of the inhibitory response. Washing out the pirenzepine for ≈1 hr caused a significant (*, $P < 0.05$) increase in the slow excitatory response (wash). Data are expressed as percentage pre-pirenzepine. Inhibitory responses were measured in mV·msec, and slow excitatory responses were measured as the increase in firing frequency over baseline for the first 5 sec of the depolarizing response. Deviation bars represent SEM.

responses by comparing the efficacy of various cholinergic agonists (Fig. 2; Table 1). Carbamoylcholine chloride (carbachol; $n = 7$), acetyl β-methacholine chloride (methacholine; $n = 11$), propionylcholine chloride ($n = 4$), and oxotremorine-M ($n = 13$) were all effective in eliciting both the hyperpolarizing and the slow voltage-dependent depolarization of pyramidal neurons (Fig. 2B; Table 1). In contrast, pilocarpine hydrochloride ($n = 9$) and suberyldicholine dichloride ($n = 5$), two cholinergic agonists that have been reported to differentially activate subtypes of muscarinic receptors (21, 32, 33), were both much more potent in eliciting the slow depolarizing response than the hyperpolarizing response (Fig. 2 E and F; Table 1), although the slow excitations elicited by pilocarpine were often relatively weak (Table 1). Application of pilocarpine to two interneurons resulted in either no response, or only a small depolarization, although application of AcCho was found to cause the typical rapid excitation (not shown).

Oxotremorine had unusual actions. Applications of this muscarinic agonist to neurons that had responded to AcCho with the typical biphasic response resulted in either a hyperpolarizing response only (Fig. 2D; $n = 4$) or a hyperpolarization followed by very weak excitation ($n = 2$). Subsequent applications of AcCho to these neurons did not evoke the slow depolarizing response, indicating that oxotremorine may in some way interfere with the ability of AcCho to elicit this response.

Applications of the ganglionic stimulant McN-A-343 (34) caused both weak inhibition and slow excitation (Table 1; $n = 10$), in agreement with pharmacological binding studies that have found this compound to be a partial muscarinic agonist that recognizes more than one muscarinic receptor subtype within the cerebral cortex (35).

DISCUSSION

Our results suggest that cholinergic inhibition and slow excitation of pyramidal cells within the cerebral cortex are mediated through different subclasses of muscarinic receptors controlling different ionic conductances on different subtypes of neurons. The direct slow excitation of pyramidal neurons is due to a voltage-dependent increase in input resistance, which has a minimum onset latency of hundreds of msec and is associated with a slow rate of membrane depolarization (only 5.1 mV/sec in tetrodotoxin or Mn^{2+}/low Ca^{2+}). A similar excitatory action of AcCho occurs in neurons of the hippocampus, olfactory cortex, and sympathetic ganglion (6–11), in which AcCho selectively diminishes a voltage-dependent K current known as the M current. In contrast, cholinergic inhibition of pyramidal neurons appears to result from a rapid excitation of inhibitory interneurons through an entirely different mechanism. Apparent cholinergic depolarization of interneurons has a relatively short onset latency, is not voltage-dependent (i.e., interneurons were depolarized by AcCho at all membrane potentials), and is associated with a decrease, and not an increase, in input resistance. These properties are like those of traditional excitatory synaptic responses. The most parsimonious explanation of our data is that AcCho acts directly on the inhibitory interneurons, although this remains to be proven. An alternative explanation for these results is that AcCho indirectly depolarizes inhibitory interneurons by causing the rapid release of another excitatory agent.

Pharmacological studies have shown that muscarinic agonists bind to receptors in the cerebral cortex in a manner that is best explained by the presence of three different binding sites [called SH, H, and L by Birdsall *et al.* (20)]. Pirenzepine is a nonclassical muscarinic antagonist that binds with ≈20 times higher affinity at the L versus the H or SH receptor sites (21, 22, 31). In the present study, pirenzepine blocked the slow depolarizing response at doses lower than those necessary to eliminate the hyperpolarizing response, suggesting that AcCho depression of the M current found in cortical pyramidal neurons may depend on activation of the L receptor sites, while the rapid excitation of interneurons may be mediated through H or SH muscarinic receptor sites. Furthermore, pirenzepine was ≈1/10th as effective in blocking the slow depolarizing response, and between 1/50th to 1/500th as effective in blocking the inhibitory response, of pyramidal neurons than atropine. These data agree well with those obtained outside of the central nervous system, where pirenzepine is 1/10th as effective as atropine in blocking AcCho action at receptors that have a high affinity for pirenzepine and ≈1/200th as effective at receptors that have a lower affinity for pirenzepine (36). The differences in sensitivity to activation by various cholinergic agents (Table

1) provide further evidence for the presence of functionally and pharmacologically distinct muscarinic receptor–effector mechanisms on cortical cells. It is not yet known whether these differences in receptor sensitivity represent an actual difference in the molecular structure of the receptor molecule itself or constraints imposed on the receptor by being coupled to different effector mechanisms.

Both the location and type of muscarinic depolarization have an important influence on the net effect of AcCho action. The slow voltage-dependent excitatory action of AcCho selectively potentiates excitatory inputs and may thereby increase the "signal-to-noise" ratio of the affected pyramidal cells (37), which mediate the output to adjacent cortex and more remote brain regions. Rapid excitation of GABAergic interneurons by AcCho may allow the cholinergic system to indirectly mediate inhibition of pyramidal neurons as well. By transiently decreasing responses to ongoing excitatory inputs, these initial inhibitory effects might serve to further increase the capacity of pyramidal neurons to respond selectively to more important inputs falling during the succeeding period of increased excitability. Alternatively, cholinergic inhibition may be used to decrease the output of selected cortical regions or neurons, while the slow depolarization increases the excitability of others. These actions of AcCho may help to explain why disruption of the telencephalic cholinergic system affects higher cognitive functions (e.g., learning and memory). The potential presence of different receptor subtypes mediating the two components of muscarinic action also raises the possibility of differential pharmacological manipulation of these responses under normal conditions or during disease states (38).

Note Added in Proof. Using extracellular unit recordings, we have found that some neurons respond with excitation to AcCho at short onset latency. This response still occurs when synaptic transmission is blocked by bathing the slices in solution containing 2 mM Mn^{2+}/0.5 mM Ca^{2+}, indicating that it is direct and not mediated through the release of other excitatory agents. Local application of scopolamine completely abolishes the response of these cells to AcCho.

We thank Dr. B. W. Connors for comments on the manuscript, J. Kadis for technical assistance, and C. Joo for help in manuscript preparation. The work in this study was supported by National Institutes of Health Grants NS 12151 and NS 06477 and the Morris Research Fund (D.A.P.). Additional support came from Huntington's Disease Foundation of America, Giannini Foundation, and National Institutes of Health fellowship NS 07331 (D.A.M.).

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