Heterogeneity of Phasic Cholinergic Signaling in Neocortical Neurons

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Gulledge AT, Park SB, Kawaguchi Y, Stuart GJ. Heterogeneity of phasic cholinergic signaling in neocortical neurons. J Neurophysiol 97: 2215–2229, 2007. First published January 10, 2007; doi:10.1152/jn.00493.2006. Acetylcholine (ACh) is a neurotransmitter critical for normal cognition. Here we demonstrate heterogeneity of cholinergic signaling in neocortical neurons in the rat prefrontal, somatosensory, and visual cortex. Focal ACh application (100 μM) inhibited layer 5 pyramidal neurons in all cortical areas via activation of an apamin-sensitive SK-type calcium-activated potassium conductance. However, subsequent data from the same laboratory found no evidence that transient mAChR activation excites interneurons in the rat neocortex (Xiang et al. 1998). On the contrary, they showed that focal ACh application (5 mM) generates muscarinic inhibition of fast-spiking (FS) interneurons, while exciting some non-FS interneurons via nicotinic receptor (nAChR) activation. We recently demonstrated an alternative mechanism for direct cholinergic inhibition of somatosensory pyramidal neurons in which transient M1-like mACHR activation releases calcium from IP3-receptor-gated intracellular stores to activate an SK-type calcium-activated potassium conductance (Gulledge and Stuart 2005). Whether this mechanism is generalized in pyramidal neurons throughout the cortex or in neocortical interneurons is not yet known.

INTRODUCTION

Although acetylcholine (ACh) is a central neurotransmitter critical for normal cognitive function, less is known about how ACh modulates the activity of individual neurons within the neocortex. Until recently, the prevailing view was that cholinergic neurons in the basal forebrain release ACh into the cortex nonspecifically and that ACh acts via volume-transmission to regulate the excitability of cortical neurons (Descarries et al. 1997). In this model, increased activity of cholinergic neurons leads to higher ambient levels of ACh and, in many cell types, increased neuronal excitability (Buzsáki et al. 1988). Recent data suggest that ACh can act in a more phasic and cell-specific manner to both increase and decrease the excitability of cortical neurons. Indeed rather than releasing ACh nonspecifically, cholinergic afferents make specialized synaptic connections with postsynaptic targets (Casu et al. 2002; Mrzljak et al. 1995; Smiley et al. 1997; Turrini et al. 2001), and extracellular concentrations of ACh in the cortex are at least an order of magnitude lower than those required to depolarize pyramidal neurons in vitro (Haj-Dahmane and Andrade 1996; Pepeu and Giovannini 2004; Vinson and Justice 1997). Finally, although increases in ACh release are correlated with activity in the cortex (Détari et al. 1999), blockade of muscarinic ACh receptors (mACHRs) in vivo can enhance some cortical responses to somatosensory stimulation (Dancoue et al. 2001). These data suggest ACh can convey a phasic signal that may excite or inhibit specific subsets of cortical neurons. A detailed understanding of how different cortical neurons respond to phasic cholinergic input is therefore critical for our understanding of how ACh facilitates information processing in the cortex.

Data demonstrating effects of transient ACh receptor activation in cortical neurons are limited. One study using guinea pig frontal cortex showed focal ACh application (1–50 mM) generated transient inhibitory responses followed by slow excitation in pyramidal neurons (McCormick and Prince 1985, 1986). The inhibitory responses were originally attributed to transient mAChR-dependent increases in the activity of GABAergic interneurons. However, subsequent data from the same laboratory found no evidence that transient mAChR activation excites interneurons in the rat neocortex (Xiang et al. 1998).

METHODS

Slice preparation

Experiments were performed using brain tissue from 3- to 5-wk-old Wistar, or 15- to 17-day-old Sprague-Dawley, rats according to

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procedures approved by the National Institute for Physiological Sciences and the Australian National University. After light anesthesia with halothane or isoflurane and decapitation, brains were removed into an ice-cold solution (ACSF) containing (in mM) 125 NaCl, 25 NaHCO₃, 3 KCl, 1.25 NaH₂PO₄, 1 CaCl₂, 5 MgCl₂, and 25 glucose (bubbled with 95% O₂-5% CO₂). Coronal slices (300 or 180 μm thick) containing medial prefrontal cortex (mPFC), somatosensory cortex, or visual cortex were cut, placed in a holding chamber filled with a similar solution (with 2 mM CaCl₂ and 1 mM MgCl₂) at room temperature and utilized for electrophysiological experiments for ≤8 h after initial preparation.

Whole cell recordings

Slices were transferred to a heated recording chamber and neurons visualized with DIC optics (Olympus BX51WI or Leica DM-LFS). Whole cell recording pipettes (5–7 MΩ) were filled with a solution containing (in mM) 135 K-glucuronate or K-methylsulfate, 7 NaCl, 2 MgCl₂, 10 HEPES, 2 Na₂ATP, and 0.3 NaGTP; pH 7.2 with KOH. No significant differences were observed in cholinergic responses in cells recorded with K-glucuronate or K-methylsulfate solutions. Data were acquired using either a BVC-700 amplifier (Dagan) or a Multiclamp 700B (Molecular Devices, Union City, CA) in current-clamp mode and a Macintosh computer (Apple Computer, Cupertino, CA) running AxoGraph X data-acquisition software (AxoGraph Scientific, Sydney, Australia). Whole cell series resistance was maximally compensated and generally between 10 and 25 MΩ. Membrane potentials were corrected for the liquid junction potential (~12 mV for K-glucuronate pipettes; ~8 mV for K-methylsulfate pipettes). Experiments were conducted at 33–35°C.

Focal drug application

For transient activation of ACh receptors, patch pipettes were filled with ACh (100 μM or 5 mM) dissolved in ACSF, connected to either a Picospritzer 3 (Parker Instrumentation, Chicago, IL), or a PV820 (World Precision Instruments, Sarasota, FL) pneumatic pump, and brief applications of ACh were applied close to the soma of recorded neurons (within ~30 μm) using a pressure of ~10 PSI. In experiments in which 5 mM ACh was applied, 2 mM kynurenic acid was co-applied to reduce glutamatergic transmission. To control for slight differences in pressure output of these devices, direct comparisons of ACh responses between drug conditions, or between cortical areas and cell types, utilized data generated with the same pneumatic device. For prolonged ACh applications to FS neurons (10 s), pipettes were withdrawn ~100 μm from the cell before triggering the application. Once triggered, the pipette was advanced to within 50 μm of the cell during the first 1–2 s of the application. During bath application of drugs, solutions were washed in for 5 min before measuring drug effects. Drugs were obtained from Sigma. GABA receptor antagonists were co-applied with kynurenic acid (2 mM).

Calcium imaging

For calcium-imaging experiments, cells were filled with the calcium-sensitive dye Oregon Green BAPTA-6F (100 μM; Invitrogen, Carlsbad, CA) for 20 min before imaging with an LSM 510 confocal microscope (Zeiss) with an Achroplan IR ×40/0.8 objective. BAPTA-6F was excited at 488 nm and the resulting fluorescence collected via a 510-nm emission filter. A single line (~1-ms duration) across the soma was scanned repeatedly at 100 Hz for 2–3 s during ACh application. Raw data were background subtracted and the change in fluorescence relative to the resting fluorescence (ΔF/ΔF₀) was calculated over a 400-ms baseline period prior to ACh application.

Immunohistochemistry

For immunohistochemistry experiments, whole cell recordings were made using thin (180 μm thick) slices from the mPFC, somatosensory, and visual cortex of 3- to 4-wk-old Wistar rats. Recordings were limited to ~15 min to reduce “washout” of soluble biochemical markers from the somata. Once finished, whole cell pipettes were slowly retracted to allow rescaling of the plasma membrane to preserve neuron integrity. Tissue slices were then fixed by immersion in a 0.1 M phosphate-buffered (PB) solution containing 4% paraformaldehyde and 0.2% picric acid. In some cases, slices were resectioned to 90 μm.

Slices were washed several times in 50 mM Tris-buffered saline (TBS) and incubated with primary antibodies for parvalbumin (PV; mouse monoclonal, Sigma; P-3171; diluted 1:2,000), somatostatin (SOM; rat monoclonal, Chemicon, MAB354; diluted 1:250), vasoactive intestinal peptide (VIP; rabbit polyclonal, Diasorin, 2007; diluted 1:2,000), or cholecystokinin (CCK; CCK/Gastrin; mouse monoclonal, #28.2 MoAb, CURE: Digestive Diseases Research Center/ Antibody and Radioimmunoassay Core; diluted 1:2,000) in TBS containing 2% bovine serum albumin, 10% normal goat serum, and 0.5% Triton-X 100. Slices were washed again in TBS and further incubated with secondary antibodies conjugated to fluorescent molecules (Alexa-594-conjugated goat anti-mouse, Alexa-594-conjugated goat anti-rat, and/or Alexa-488-conjugated goat anti-rabbit; Sigma; diluted 1:200) and Alexa-530-streptavidin (Molecular Probes). Once washed, cells were visualized using a fluorescence microscope (Olympus BX60).

Data analysis

Data throughout are presented as means ± SD. Except as noted, statistical analysis used either the Student’s t-test (2-tailed, paired or unpaired) or an ANOVA with a Tukey-Kramer multiple comparisons posttest. Non-FS neurons were classified into two groups based on the presence or absence of a hyperpolarization-activated cationic current ("I_h"), measured long (1.5 s) hyperpolarizing current injections that produced a peak hyperpolarization of ~30 mV from the resting potential (mean change in membrane potential, V_M, was ~29 ± 8; n = 62). The amount of “sag,” indicative of I_h, was quantified as the peak hyperpolarization divided by the amplitude of the steady-state potential measured relative to the peak hyperpolarization. Cells were considered to have I_h if the amount of sag was ≥15% (see supplemental Fig. 1), and both "I_h-positive" and "I_h-negative" neurons were exposed to similar hyperpolarizing steps (mean of each was ~29 ± 8 mV; n = 62; P = 0.86).

RESULTS

Muscarinic inhibition is a general feature of layer 5 pyramidal neurons

To determine whether cholinergic inhibition is a general feature of neocortical pyramidal neurons, we compared the effects of transient ACh receptor activation (100 μM ACh applied for 20 ms) in pyramidal neurons from different cortical layers in 3 cortical regions: the mPFC, somatosensory, and visual cortex (Fig. 1). Cholinergic inhibition of pyramidal neurons was generally confined to cells in deeper cortical layers, with almost all layer 5 pyramidal neurons hyperpolarized by ACh (29 of 30; mean response amplitude = −4.4 ± 3.0 mV; n = 29). Responses in layer 5 mPFC neurons (n = 10) were significantly larger than those in somatosensory (n = 10) or visual cortex (−7.0 ± 3.4 mV in mPFC vs. −2.5 ± 1.4 mV in somatosensory and −3.6 ± 1.4 mV in visual cortex; n = 29 total cells; P < 0.001, ANOVA). Superficial layer 2/3 pyra-
midal neurons within 125 μm of layer 1 had fewer responding neurons (6 of 23 cells) with smaller individual responses in those neurons that did show cholinergic inhibition (mean response = −1.2 ± 1.2 mV, n = 6; P < 0.01 when compared with responses in layer 5). Neurons deeper in cortical layer 3 had heterogeneous responses that depended on cortical area. In mPFC and somatosensory cortex, deeper neurons in layer 3 (those >125 μm from layer 1) were less responsive than layer 5 neurons in the same cortical areas with only 8 of 16 neurons exhibiting cholinergic inhibition (mean response = −2.5 ± 3.4 mV, n = 8). In contrast, neurons in deeper layer 3 of visual cortex had robust responses (mean amplitude = −5.3 ± 3.9 mV, n = 7). With failures (0 amplitude) included, cholinergic responses in layer 2/3 neurons were significantly dependent on their distance from layer 1 (r = 0.49, P < 0.001; Spearman rank correlation). Further, cholinergic responsiveness in layer 3 of visual cortex was significantly greater than in prefrontal or somatosensory cortex (P < 0.05, ANOVA). To confirm the lack of cholinergic responses in layer 2/3 neurons, we applied ACh for 200 ms. In only one case did the longer ACh application reveal a hyperpolarizing response (a somatosensory cortex layer 3 neuron; −1.9 mV response). As summarized in Fig. 1B, these data demonstrate that phasic ACh application preferentially inhibits deeper-layer pyramidal neurons, while having a greatly reduced efficacy in the most superficial cells. Because layer 5 neurons provide the main output of the neocortex, these data suggest that ACh may inhibit cortical output while permitting intracortical processing in superficial pyramidal neurons.

ACh cellular signaling in pyramidal neurons is similar across cortical areas

Cholinergic inhibition in layer 5 pyramidal neurons in somatosensory cortex depends on activation of an apamin-sensitive SK-type calcium-activated potassium conductance (Gulledge and Stuart 2005). To confirm that SK channels play a role in cholinergic inhibition throughout the cortex, in a different set of experiments, we focally applied ACh (100 μM, 50 ms) to pyramidal neurons in mPFC (layer 5, n = 6), somatosensory (layer 5, n = 9), and visual cortex (layer 5, n = 4; layer 3, n = 3) before and after bath application of apamin (100 nM). Application of ACh resulted in hyperpolarizing responses in all neurons tested (Fig. 2A) with responses in prefrontal neurons being of somewhat larger in amplitude and duration than in neurons in other areas of cortex (Fig. 2B; ANOVA, P < 0.01 and P < 0.05 for amplitude and 1/2-width, respectively, when compared with responses in somatosensory neurons; Table 1). Bath application of apamin completely blocked all hyperpolarizing responses (mean response in apamin = +1.6 ± 1.6 mV, n = 22), suggesting a common ionic mechanism mediates cholinergic inhibition in pyramidal neurons throughout the cortex (Fig. 2B).

FIG. 1. Comparison of transient cholinergic responses in pyramidal neurons in multiple cortical regions and layers. A: representative responses of neocortical pyramidal neurons in different layers of the medial prefrontal cortex (mPFC), somatosensory, and visual cortex to acetylcholine (ACh) application (100 μM, 20 ms). B: summary plot showing response amplitude vs. distance from layer 1 for data from neurons in the mPFC (blue), somatosensory (green), and visual cortex (black). Data from neurons in which hyperpolarizing responses did not occur are outlined in red.
mAChR antagonist pirenzepine (0.5 or 1 mM) to prefrontal layer 5 pyramidal neurons receiving focal ACh applications (Fig. 3, A and B). In baseline conditions for these three experimental groups, ACh applications (15–20 ms) generated inhibitory responses of \(-6.2 \pm 1.6 \text{ mV} (n = 6), -4.9 \pm 1.4 \text{ mV} (n = 5), \text{ and } -6.1 \pm 2.0 \text{ mV} (n = 5)\), respectively. Bath application of atropine or pirenzepine completely abolished responses to ACh (mean responses to ACh in the presence of antagonists were 0.2 \pm 0.1 and 0.1 \pm 0.3 mV, respectively), whereas methoctramine caused only a slight reduction in response amplitude (mean response in methoctramine = -4.5 \pm 1.3 mV; Fig. 3B). These data demonstrate that cholinergic inhibition in prefrontal neurons requires M1-like receptor activation.

We next tested whether cholinergic inhibition in prefrontal pyramidal neurons depends on GABAergic synaptic transmission. In the presence of GABA_A (picrotoxin, 100 \mu M, and SR95531, 10 \mu M) and GABA_B (CGP55845, 1 \mu M) antagonists, ACh (10–20 ms) generated inhibitory responses of \(-4.8 \pm 2.1 \text{ mV} (n = 10)\); values not significantly different from control experiments in the absence of GABAergic antagonists (Fig. 3C; mean response = -4.5 \pm 1.6 mV, n = 10). Additionally, experiments in which ACh was applied during manipulations of the somatic membrane potential found ACh responses to have reversal potentials near the estimated potassium equilibrium potential of \(-98 \text{ mV} (E_{\text{K}} = -95.7 \pm 1.7 \text{ mV, } n = 6); \text{ data not shown).}

The preceding data are consistent with a direct cholinergic inhibition of pyramidal neurons that relies on activation of a potassium conductance. We next confirmed that calcium signaling is a necessary step in generating this apamin-sensitive conductance. As previously described in somatosensory cortex (Gulledge and Stuart 2005), inhibitory responses in prefrontal neurons were completely abolished when the calcium-chelating agent BAPTA (10 \mu M) was included in the pipette solution (n = 9, including 2 neurons that were patched twice, with BAPTA only in the 2nd pipette; Fig. 3D).

We further confirmed that the necessary calcium signaling in prefrontal neurons is mediated by calcium release from intracellular stores by bath applying cyclopiazonic acid (CPA, 30 \mu M) to deplete stored calcium. CPA significantly reduced ACh-induced hyperpolarizations from \(-4.3 \pm 0.6 \text{ mV} \text{ in baseline conditions to } -0.7 \pm 0.8 \text{ mV} \text{ in CPA (n = 4; } P < 0.001; \text{ data not shown). To determine whether IP_3 or ryanodine receptors are necessary for cholinergic inhibition, we performed additional experiments in which either heparin (2–3 mg/ml), an IP_3 receptor antagonist, or ruthenium red (40 \mu M), a ryanodine receptor antagonist, was included in the pipette saline. The presence of heparin caused a progressive decrease in response amplitude over several minutes such that responses

![Fig. 2. Cholinergic inhibition results from SK-type channel activation. A: representative responses of layer 5 neocortical pyramidal neurons to brief applications of ACh (100 \mu M, 50 ms). Top: baseline responses of neurons from the mPFC (left), somatosensory cortex (middle), and visual cortex (right). Bottom: responses recorded after 5 min exposure to apamin (100 nM). B: summary graph comparing mean responses of layer 5 pyramidal neurons from the mPFC and somatosensory cortex and combined data from cells in layer 3 (n = 3) and layer 5 (n = 4) of the visual cortex. All neurons tested had hyperpolarizing responses to ACh that were completely abolished by apamin application (n = 22), with control responses in prefrontal neurons being significantly larger than those in somatosensory cortex (P < 0.01, ANOVA). Data shown as means ± SD.

Although the data from our apamin experiments show that SK channels are involved in generating cholinergic inhibition in pyramidal neurons, we wanted to confirm that the signaling cascade previously described in somatosensory neurons also mediates inhibitory responses in pyramidal neurons in other cortical areas. Because cholinergic responses were somewhat larger and more prolonged in prefrontal pyramidal neurons, we conducted additional experiments to define the signal transduction mechanisms mediating cholinergic inhibition in these cells (Fig. 3). We first tested whether cholinergic inhibition requires activation of M1- or M2-like mAChRs by bath-applying the nonspecific mAChR antagonist atropine (1 \mu M), the M1-like mAChR antagonist pirenzepine (0.5 or 1 \mu M), or the M2-like mACHR antagonist methoctramine (1 \mu M) to prefrontal layer 5 pyramidal neurons receiving focal ACh applications (Fig. 3, A and B). In baseline conditions for these three experimental groups, ACh applications (15–20 ms) generated inhibitory responses of \(-6.2 \pm 1.6 \text{ mV} (n = 6), -4.9 \pm 1.4 \text{ mV} (n = 5), \text{ and } -6.1 \pm 2.0 \text{ mV} (n = 5)\), respectively. Bath application of atropine or pirenzepine completely abolished responses to ACh (mean responses to ACh in the presence of antagonists were 0.2 \pm 0.1 and 0.1 \pm 0.3 mV, respectively), whereas methoctramine caused only a slight reduction in response amplitude (mean response in methoctramine = -4.5 \pm 1.3 mV; Fig. 3B). These data demonstrate that cholinergic inhibition in prefrontal neurons requires M1-like receptor activation.

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**TABLE 1. Hyperpolarizing responses in neocortical pyramidal neurons**

<table>
<thead>
<tr>
<th>Cortical Area</th>
<th>n</th>
<th>V_M, mV</th>
<th>Amplitude, mV</th>
<th>Latency, ms</th>
<th>Rise, ms</th>
<th>Half Width, ms</th>
</tr>
</thead>
<tbody>
<tr>
<td>mPFC</td>
<td>6</td>
<td>-79 ± 4</td>
<td>-6.2 ± 1.2**</td>
<td>286 ± 102</td>
<td>408 ± 340</td>
<td>1311 ± 775*</td>
</tr>
<tr>
<td>Somatosensory cortex</td>
<td>9</td>
<td>-77 ± 3</td>
<td>-3.5 ± 1.0**</td>
<td>368 ± 86</td>
<td>233 ± 134</td>
<td>590 ± 235*</td>
</tr>
<tr>
<td>Visual cortex</td>
<td>7</td>
<td>-81 ± 3</td>
<td>-4.5 ± 2.1</td>
<td>331 ± 24</td>
<td>230 ± 87</td>
<td>914 ± 214</td>
</tr>
<tr>
<td>Combined areas</td>
<td>22</td>
<td>-79 ± 3</td>
<td>-4.5 ± 1.8</td>
<td>334 ± 81</td>
<td>280 ± 207</td>
<td>890 ± 516</td>
</tr>
</tbody>
</table>

V_M, resting potential. Data are for local acetylcholine (ACh) applications (100 \mu M; 50 ms) to layer 5 neurons in the medial prefrontal cortex (mPFC) and somatosensory cortex, and layer 5 (n = 4) and layer 3 (n = 3) neurons from visual cortex. Significant differences between groups indicated as * = P < 0.05 and ** = P < 0.01, one-way ANOVA.
after 10 min were reduced to 17 ± 7% of the original amplitude (Fig. 3E; 1st ACh application given just after whole cell "break in" with subsequent applications at 15-s intervals). Initial responses to ACh were −4.8 ± 1.2 mV, decreasing to −0.8 ± 0.4 mV after 10 min with heparin in the pipette ($P < 0.0001$). On the other hand, no reduction in response amplitude was observed when ruthenium red was included in the pipette (response after 10 min = −5.0 ± 2.2 mV, $n = 5$) or in control neurons patched with regular internal saline (response after 10 min = −6.6 ± 0.7 mV; $n = 4$).

Finally, to confirm that ACh application leads to an increase in intracellular calcium, we filled additional neurons with the calcium-sensitive dye Oregon Green BAPTA-6F (100 μM) and used confocal microscopy to detect changes in intracellular calcium at the soma. Brief applications of ACh (10 ms) generated coincident hyperpolarizing responses and increases in fluorescence in seven of seven neurons tested (Fig. 3F). In these experiments, peak $\Delta F/F$ ratios of 1.0 ± 0.3 were generated in response to ACh application at rest and during action potential firing generated by somatic current injection. In five of these imaging experiments, we bath-applied the cholinergic agonist carbamoyl (10 μM). ACh applied in the presence of tMACHR activation with carbamoyl was still able to generate increases in intracellular calcium and membrane hyperpolarization in four of five neurons (mean change in $\Delta F/F$ was $+0.4 \pm 0.3, n = 4$) even during periods of carbamoyl-induced spontaneous action potential firing (Fig. 3F). These data confirm that cholinergic inhibition of prefrontal pyramidal neurons relies on the same intracellular signaling processes employed in somatosensory layer 5 neurons, and suggest that a M1-like receptor – IP$_3$ – Ca$^{2+}$ – SK-channel pathway is a common mechanism for cholinergic inhibition of neocortical pyramidal neurons. Further, the data confirm phasic cholinergic inhibition can occur even during periods of excitation via tMACHR activation.

**Role of action potentials in promoting cholinergic inhibitory responses**

Although cholinergic inhibitory responses to repeated ACh applications show rapid rundown in amplitude, rundown can be prevented by short periods of action potential generation between agonist applications (Gulledge and Stuart 2005). To determine the relationship between action potentials and response recovery, we applied ACh to neurons after conditioning them with a variable number of action potentials (0–150 spikes; Fig. 4A). In these experiments, neurons were first exposed to a preconditioning train of 150 action potentials generated by brief somatic current injections (3 nA for 4 ms at 25 Hz), and then inhibitory cholinergic responses were “run-down” with a series of 3 ACh applications (50 ms) delivered at 0.5 Hz. This procedure produced substantial rundown of the cholinergic response, as indicated by diminished responses to a test application of ACh 10.5 s later (responses reduced to 19 ± 30% of control values, $n = 10$; $P < 0.01$; 0-spikes condition shown in Fig. 4, B and C). To determine the number of spikes necessary for response facilitation, we applied conditioning trains of action potentials of varying duration (current injection of 3 nA for 4 ms at 25 Hz; 0, 5, 10, 20, 50, 100, or 150 spikes applied 2.5 s after the 3rd control ACh application). The summary data for these experiments show that trains of ≥50 action potentials are required to significantly facilitate inhibitory responses to subsequent ACh application (Fig. 4C). Interestingly, when neurons experienced a second train of 150 spikes generated shortly (2.5 s) after ACh exposure, responses to test applications of ACh were significantly larger than the
response to the first control application of ACh (test response was 135 ± 27% of the amplitude of the 1st of the 3 control ACh applications; P < 0.01; Fig. 4, B and C). These data suggest that ≥50 action potentials are needed to facilitate inhibitory cholinergic responses and that previous exposure to ACh can enhance the ability of action potentials to prime neurons for subsequent mAChR-mediated inhibitory responses.

Temporal and spatial characteristics of cholinergic inhibition

As demonstrated in Fig. 3F transient mAChR activation generates inhibitory responses in neurons even during periods of action potential firing. Indeed, action potential firing facilitates cholinergic inhibition and prevents the rundown of responses during repetitive ACh applications (Gulledge and Stuart 2005). To determine how rapidly cortical pyramidal neurons can follow repetitive cholinergic input, we depolarized somatosensory and prefrontal layer 5 pyramidal neurons and applied ACh (20 ms) multiple times at several application frequencies (8–15 applications at 0.2–1 Hz during a 40-s test period; Fig. 5A). To identify periods of ACh-induced inhibition during high-frequency spike trains, we plotted instantaneous spike frequency (ISF) over time (Fig. 5A) and identified neurons that were able to follow trains of ACh applications as those in which a discrete reduction in ISF was observed for each of the first 8 or 10 consecutive applications (only 8 applications were given at 0.2 Hz in the 40-s trial). After the first application of ACh, there was generally a rapid reduction in the ability of subsequent ACh applications to reduce ISF with inhibitory responses reaching a frequency-dependent steady-state level after two or three exposures to ACh (Fig. 5B). As summarized in Fig. 5C, all neurons tested were able to follow ACh applications at 0.2 and 0.25 Hz with substantial failures to follow repetitive ACh exposure beginning at 0.5 Hz (only 50% of cells able to follow 10 consecutive ACh applications). Because synaptic release of ACh in vivo is expected to be more rapid and receptor-targeted than exogenous drug application, it is likely that the focal applications of ACh used in these experiments underestimate the ability of pyramidal neurons to follow repetitive ACh release.

In all of the experiments in the preceding text, we applied ACh to the soma and proximal dendrites of pyramidal neurons (within ~30 μm). In a separate set of experiments, we tested whether ACh applied at different locations along the apical dendrite can also generate inhibitory responses in these neurons (Fig. 6). Recording from pyramidal neurons from the mPFC and somatosensory cortex, we focally applied ACh (50 ms) to the soma and at several locations along the apical dendrite (50, 100, 150, and 300 μm from the soma). Although ACh consistently generated inhibitory responses at the soma, the ability of ACh to hyperpolarize neurons was significantly reduced even at the closest dendritic location (50 μm; ANOVA, P < 0.01), with responses being reduced to 9 ± 13% of baseline values at a distance of 150 μm from the soma (Fig. 6B). Although some dendritic filtering of the inhibitory response would be expected, the attenuation observed at 150 μm cannot be explained simply by cable properties of the apical dendrite as steady-state attenuation at this distance is <30% (Gulledge and Stuart 2003; Stuart and Spruston 1998). In a subset of these experiments, we applied ACh for a prolonged period of time (20 s) within layer 1 (Fig. 6A). No significant change in membrane potential was measurable at the soma (mean change after 20 s = 0.1 ± 0.2 mV, n = 8). Together these data suggest that cholinergic inhibition in layer 5 pyramidal neurons is generated close to the somata of these cells.

ACh does not modulate the excitability of FS interneurons

The preceding data demonstrate that transient ACh receptor activation preferentially inhibits the output of layer 5 pyramidal neurons while having a reduced inhibitory effect in superficial pyramidal neurons. The neocortex also contains a large number of inhibitory, nonpyramidal interneurons (Kawaguchi and Kubota 1997). Although data regarding the effects of focal ACh application on interneurons are limited, there is evidence that transient mAChR activation reduces the excitability of the FS neurons that...
provide the bulk of perisomatic inhibition to pyramidal neurons (Xiang et al. 1998). In contrast, nicotinic receptor-mediated excitatory responses, which are minimal in pyramidal neurons, have been demonstrated in certain groups of non-FS neurons (Christophe et al. 2002; Porter et al. 1999; Xiang et al. 1998).

To clarify the relative impact of muscarinic and nicotinic signaling in interneurons, we focally applied ACh to FS and non-FS neurons in mPFC, somatosensory, and visual cortex. FS cells were easily identified based on their physiological properties, including a relatively low input resistance ($R_N$; mean value was $163 \pm 86$ M$\Omega$, $n = 37$) and trains of action potentials that show little if any frequency adaptation, reduction in action potential amplitude, increase in spike half-width, or changes in afterhyperpolarization amplitude (Fig. 7A).

When ACh (100 $\mu$M; 50 – 200 ms) was focally applied, hyperpolarizing responses were observed in the majority of FS neurons (19 of 31 FS neurons; Tables 2 and 3). However, bath-applied atropine (1 $\mu$M) failed to block or significantly reduce hyperpolarizing responses ($n = 4$ of 4 neurons tested), suggesting again that mAChRs are not involved in their generation (mean response to 5 mM ACh in baseline conditions was $-1.5 \pm 0.4$ mV; mean response in atropine was $-1.3 \pm 0.3$ mV, $n = 4$; $P = 0.25$). In summary, although hyperpolarizing responses to ACh (0.1 or 5 mM) were observed in 24 of 37 FS cells, they were not sensitive to atropine application ($n = 10$).

Although only one study has previously described mAChR-mediated hyperpolarization in FS neurons (Xiang et al. 1998), the same laboratory has also reported that focally applied serotonin (5-HT; 100 $\mu$M) produces an identical hyperpolarizing response in these cells (Xiang and Prince 2003). In a separate experiment, we tested the ability of 5-HT (100 $\mu$M) to produce hyperpolarizing responses in seven FS neurons and observed hyperpolarizing responses in four cells (Table 3). In two of these neurons, the ability of focal 5-HT application to generate hyperpolarizing responses was challenged with bath-application of the 5-HT antagonists WAY-100635 (5 $\mu$M) and ritanserin (2 $\mu$M). These antagonists failed to block hyperpolarizing responses to 5-HT application (data not shown).
Because our pharmacological manipulations were unable to block hyperpolarizing responses in FS neurons, we speculated that focal application itself might be producing the response. To test this, we puffed drug-free ACSF (50–200 ms) onto FS neurons \((n = 12)\), non-FS cells \((n = 8)\), and pyramidal neurons \((n = 12)\). Although focal application of ACSF produced hyperpolarizing responses in 8 of 12 FS neurons (Fig. 7C1), no responses of any kind were observed in non-FS cells or in pyramidal neurons (Fig. 7C2 and C3). The fraction of neurons showing hyperpolarizing responses to focal application of ACSF (67%) was similar to the fraction of neurons hyperpolarized by ACh or 5-HT (27 of 44; 61%). Furthermore no significant differences were found in the amplitude \((P = 0.73)\), latency \((P = 0.40)\), rise time \((P = 0.98)\), and half-widths \((P = 0.92)\) of ACSF- and ACh-induced hyperpolarizing responses in FS cells \((n = 31)\;\text{Table 3}\). When compared with ACh-induced hyperpolarizing responses from a sample of pyramidal (see Table 1) and non-FS neurons (see following text), hyperpolarizing responses in FS neurons were smaller in amplitude and.

![Figure 6](http://jn.physiology.org/)

**FIG. 6.** Cholinergic inhibition is restricted to ACh applications near the soma. A: representative responses in a prefrontal neuron to ACh (50 ms) applied to the soma and at various locations along the apical dendrite. Top: trace during a long-duration (20 s) application of ACh in layer 1 directly above the apical dendrite of the layer 5 neuron. The 2 traces at the somatic location were collected before and after the ACh applications to the apical dendrite, showing that decreased responsiveness in the dendrite is not due to rundown of cholinergic signaling over time. B: summary plot comparing response amplitudes for ACh delivered to apical dendritic locations relative to the response to ACh applied near the soma. Gray lines and points show data from individual neurons. Black lines and filled circles show mean responses across cells \(\pm \text{SD}\) \((n = 10; \text{neurons from mPFC and somatosensory cortex})\).

![Figure 7](http://jn.physiology.org/)

**FIG. 7.** Transient ACh application does not modulate fast-spiking (FS) neurons. A: membrane responses to subthreshold positive and negative current injections and the resulting \(V-I\) plot (left) and suprathreshold responses (right) for a FS neuron in the mPFC. Inset is an enlargement of the initial 5 action potentials showing that all spikes have a similar spike amplitude, width, afterhyperpolarization (AHP) size, and inter-spike interval. B: averaged trace of 4 consecutive trials showing an atropine-insensitive hyperpolarizing response to focal ACh application in an FS neuron from the mPFC. Data from 5 representatives neurons in each group are shown. C: pressure application of drug-free saline alone [artificial cerebrospinal fluid (ACSF)] generates hyperpolarizing responses selectively in FS neurons. Shown are average traces of 4 consecutive trials in which ACSF was applied to FS (C1), non-FS (C2), and pyramidal neurons (C3). Data from 5 representative neurons in each group are shown. D: responses in 2 FS neurons to prolonged (10 s) applications of ACh (100 \(\mu\)M in the presence of 1 \(\mu\)M atropine) or ACSF.
TABLE 2.  Cholinergic responses in neocortical interneurons

<table>
<thead>
<tr>
<th>Cell Type</th>
<th>Area, Cell Group</th>
<th>Layer</th>
<th>n</th>
<th>Vm, mV</th>
<th>Rm, MΩ</th>
<th>Response to Transient ACh Application</th>
<th>Puff artifact (Δ VM)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td>No Effect</td>
<td>nACHR</td>
</tr>
<tr>
<td>FS cells</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>PFC</td>
<td></td>
<td>3</td>
<td>4</td>
<td>−67 ± 4</td>
<td>121 ± 34</td>
<td>4</td>
<td>100</td>
</tr>
<tr>
<td></td>
<td></td>
<td>5</td>
<td>16</td>
<td>−79 ± 6</td>
<td>165 ± 86</td>
<td>16</td>
<td>100</td>
</tr>
<tr>
<td>SS cortex</td>
<td></td>
<td>3</td>
<td>3</td>
<td>−81 ± 3</td>
<td>103 ± 31</td>
<td>3</td>
<td>100</td>
</tr>
<tr>
<td>Vis cortex</td>
<td></td>
<td>5</td>
<td>4</td>
<td>−77 ± 5</td>
<td>250 ± 128</td>
<td>4</td>
<td>100</td>
</tr>
<tr>
<td></td>
<td></td>
<td>5</td>
<td>6</td>
<td>−77 ± 4</td>
<td>201 ± 73</td>
<td>6</td>
<td>100</td>
</tr>
<tr>
<td>Total layer 3</td>
<td></td>
<td>11</td>
<td>75</td>
<td>106 ± 27</td>
<td>11</td>
<td>100</td>
<td>0</td>
</tr>
<tr>
<td>Total layer 5</td>
<td></td>
<td>26</td>
<td>78</td>
<td>5 ± 25</td>
<td>26</td>
<td>100</td>
<td>0</td>
</tr>
<tr>
<td>All FS cells</td>
<td></td>
<td>37</td>
<td>77</td>
<td>6 ± 100</td>
<td>37</td>
<td>100</td>
<td>0</td>
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<td>Non-FS cells</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>PFC</td>
<td></td>
<td>3</td>
<td>12</td>
<td>−78 ± 4</td>
<td>250 ± 92</td>
<td>2</td>
<td>17</td>
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<tr>
<td></td>
<td></td>
<td>5</td>
<td>10</td>
<td>−74 ± 8</td>
<td>319 ± 80</td>
<td>6</td>
<td>60</td>
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<tr>
<td>SS cortex</td>
<td></td>
<td>3</td>
<td>8</td>
<td>−74 ± 7</td>
<td>246 ± 98</td>
<td>6</td>
<td>75</td>
</tr>
<tr>
<td>Vis cortex</td>
<td></td>
<td>5</td>
<td>6</td>
<td>−77 ± 4</td>
<td>331 ± 114</td>
<td>2</td>
<td>33</td>
</tr>
<tr>
<td></td>
<td></td>
<td>3</td>
<td>4</td>
<td>−70 ± 2</td>
<td>185 ± 18</td>
<td>4</td>
<td>100</td>
</tr>
<tr>
<td>Total layer 3</td>
<td></td>
<td>24</td>
<td>75</td>
<td>6 ± 100</td>
<td>24</td>
<td>100</td>
<td>0</td>
</tr>
<tr>
<td>Total layer 5</td>
<td></td>
<td>36</td>
<td>76</td>
<td>5 ± 100</td>
<td>36</td>
<td>100</td>
<td>0</td>
</tr>
<tr>
<td>All non-FS cells</td>
<td></td>
<td>60</td>
<td>76</td>
<td>6 ± 100</td>
<td>60</td>
<td>100</td>
<td>0</td>
</tr>
</tbody>
</table>

FS, fast-spiking interneuron; non-FS = non-fast-spiking interneuron; PFC, medial prefrontal cortex; SS cortex, somatosensory cortex; Vis cortex, visual cortex; Vm, resting potential; Rm, input resistance; nACHR, nicotinic response to ACh; pressure artifact, hyperpolarization induced by pressure drug application. *Cells showing only artifacts are included in the “no effect” category. ACh was locally applied at a 100 μM concentration, except for 6 FS and 12 non-FS cells to which 5 mM ACh was applied. Data shown as means ± SD.

TABLE 3.  Comparison of pressure artifacts in FS cells with cholinergic responses in non-FS and pyramidal neurons

<table>
<thead>
<tr>
<th>Cell Type</th>
<th>Drugs Applied</th>
<th>Cells Tested</th>
<th>Cells With Response</th>
<th>Percentage</th>
<th>Amplitude, mV</th>
<th>Latency, ms</th>
<th>Rise Time, ms</th>
<th>Half Width, ms</th>
</tr>
</thead>
<tbody>
<tr>
<td>FS cells (Wistar)</td>
<td>ACh (100 μM)</td>
<td>31</td>
<td>19</td>
<td>61</td>
<td>−1.9 ± 1.2</td>
<td>90 ± 57</td>
<td>91 ± 95</td>
<td>578 ± 651</td>
</tr>
<tr>
<td></td>
<td>ACh (5 mM)</td>
<td>6</td>
<td>4</td>
<td>67</td>
<td>−1.5 ± 0.4</td>
<td>51 ± 41</td>
<td>97 ± 90</td>
<td>260 ± 44</td>
</tr>
<tr>
<td></td>
<td>ACh + atropine</td>
<td>10*</td>
<td>10</td>
<td>100</td>
<td>−1.8 ± 1.1</td>
<td>52 ± 51</td>
<td>71 ± 49</td>
<td>344 ± 240</td>
</tr>
<tr>
<td></td>
<td>5-HT (100 μM)</td>
<td>7</td>
<td>4</td>
<td>57</td>
<td>−2.0 ± 0.9</td>
<td>67 ± 21</td>
<td>70 ± 16</td>
<td>274 ± 176</td>
</tr>
<tr>
<td></td>
<td>ACSF</td>
<td>12</td>
<td>8</td>
<td>67</td>
<td>−1.7 ± 0.6</td>
<td>50 ± 105</td>
<td>113 ± 81</td>
<td>501 ± 390</td>
</tr>
<tr>
<td>FS cells (Sprague-Dawley)</td>
<td>ACh (5 mM)</td>
<td>6</td>
<td>5</td>
<td>83</td>
<td>−0.9 ± 0.4</td>
<td>48 ± 34</td>
<td>253 ± 171</td>
<td>4118 ± 3271</td>
</tr>
<tr>
<td></td>
<td>ACh + Scp + Atr</td>
<td>5*</td>
<td>5</td>
<td>100</td>
<td>−0.9 ± 0.3</td>
<td>34 ± 11</td>
<td>200 ± 102</td>
<td>1664 ± 2014</td>
</tr>
<tr>
<td></td>
<td>ACSF</td>
<td>4</td>
<td>3</td>
<td>75</td>
<td>−1.3 ± 2.2</td>
<td>43 ± 6</td>
<td>157 ± 17</td>
<td>1508 ± 332</td>
</tr>
<tr>
<td></td>
<td>ACSF + TTX</td>
<td>3*</td>
<td>3</td>
<td>100</td>
<td>−1.5 ± 0.4</td>
<td>36 ± 12</td>
<td>185 ± 86</td>
<td>1735 ± 359</td>
</tr>
<tr>
<td>Non-FS cells (Wistar)</td>
<td>ACh (100 μM)</td>
<td>60</td>
<td>4</td>
<td>7</td>
<td>−4.7 ± 1.0</td>
<td>295 ± 196</td>
<td>419 ± 294</td>
<td>1595 ± 1171</td>
</tr>
<tr>
<td></td>
<td>ACh + apamin</td>
<td>3*</td>
<td>3</td>
<td>100</td>
<td>−5.3 ± 1.2</td>
<td>368 ± 254</td>
<td>430 ± 283</td>
<td>1442 ± 910</td>
</tr>
<tr>
<td></td>
<td>ACh + atropine</td>
<td>4*</td>
<td>0</td>
<td>0</td>
<td>0.0 ± 0.2*</td>
<td>0</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td></td>
<td>(Group 2)</td>
<td>12†</td>
<td>12</td>
<td>100</td>
<td>−7.3 ± 2.5</td>
<td>268 ± 197</td>
<td>339 ± 257</td>
<td>1297 ± 599</td>
</tr>
<tr>
<td></td>
<td>ACh + MCT</td>
<td>6*</td>
<td>5</td>
<td>100</td>
<td>−0.9 ± 0.6‡</td>
<td>505 ± 220</td>
<td>152 ± 74</td>
<td>300 ± 272‡</td>
</tr>
<tr>
<td></td>
<td>ACh + PZP</td>
<td>6*</td>
<td>6</td>
<td>100</td>
<td>−4.3 ± 3.9</td>
<td>361 ± 222</td>
<td>311 ± 173</td>
<td>979 ± 478‡</td>
</tr>
<tr>
<td></td>
<td>ACSF</td>
<td>8</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>Pyramidal Neurons</td>
<td>ACh (100 μM)</td>
<td>22‡</td>
<td>22</td>
<td>100</td>
<td>−4.5 ± 1.8</td>
<td>334 ± 81</td>
<td>280 ± 207</td>
<td>890 ± 516</td>
</tr>
<tr>
<td></td>
<td>ACSF</td>
<td>12</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>0</td>
</tr>
</tbody>
</table>

5-HT, serotonin; ACSF, standard recording solution (no drug); Scp, scopolamine (100 μM); Atr, atropine (1 μM); MCT, methoctramine (500 nM); PZP, pirenzepine (500 nM). Data shown as means ± SD. An asterisk (*) indicates drugs applied to subsets of cells already showing hyperpolarizing responses to ACh or ACSF application. TTX was applied at 1 μM. †Cells tested in a second group of specifically-targeted non-FS cells from the mPFC that showed hyperpolarization to ACh; 6 of these neurons were then exposed to MCT, and another 6 exposed to PZP. ‡Indicates significant differences in the presence of antagonists (P < 0.05). §Combined data from pyramidal neurons shown in Table 1.

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FIG. 8. ACh does not modulate FS cells from the visual cortex of young (15- to 17-day-old) SD rats. A1: response of a visual cortex FS neuron to focal ACh application (5 mM; 200 ms) before (top) and after (bottom) bath application of a mixture of 2 muscarinic antagonists (scopolamine, 100 µM, and atropine, 1 µM). Traces are averages of 6–8 consecutive trials. A2: in a different neuron, response to focal application of drug-free ACSF (200 ms) before (top) and after (bottom) bath application of TTX (1 µM). Traces are averages of 6 consecutive trials. B1: summary of responses to 5 mM ACh in SD FS neurons before and after mAChR antagonist applications (n = 5). B2: summary (mean ± SD) of hyperpolarizing responses to drug-free ACSF before and after TTX application (n = 3). Scale bars are the same in A1 and 2.

Together, the preceding data suggest that transient ACh exposure does not modulate the excitability of FS neurons via mAChR activation but rather that focal application itself can cause hyperpolarizing responses in FS cells. However, because the sole previous report of cholinergic inhibition of FS cells examined neurons in the visual cortex from young (12- to 17-day old) Sprague-Dawley (SD) rats (Xiang et al., 1998), it is possible that cholinergic inhibition of FS cells is age or animal strain dependent. To confirm that this is not the case, we performed additional experiments applying ACh (5 mM) or ACSF to FS neurons from the visual cortex of 15- to 17-day-old SD rats (Fig. 8). Focal ACh application induced hyperpolarizing responses in five of six FS neurons (mean amplitude = −0.9 ± 0.4 mV) that were completely insensitive to bath application of a combination of cholinergic antagonists (100 µM scopolamine plus 1 µM atropine; mean amplitude in antagonists was −0.9 ± 0.3 mV; n = 5; Fig. 8, A and C).

Further, focal application of drug-free ACSF generated hyperpolarizing responses in three of four FS neurons tested (amplitude of −1.3 ± 0.2 mV; n = 3; Fig. 8B). Responses to focal ACSF application were not sensitive to bath application of TTX (1 µM; mean amplitude in TTX = −1.5 ± 0.4 mV; n = 3; Fig. 8D), arguing against an indirect effect of ACSF application on synaptic release of other transmitters. Together these data suggest ACh does not directly modulate FS neuron excitability.

**TABLE 4. Cholinergic responses in non-FS interneurons in layers 2/3 and 5**

<table>
<thead>
<tr>
<th>Cell Group</th>
<th>n</th>
<th>V_m (mV)</th>
<th>R_N (MΩ)</th>
<th>Sag</th>
<th>Percentage</th>
<th>Decrease in V_m</th>
<th>n</th>
<th>Percentage</th>
</tr>
</thead>
<tbody>
<tr>
<td>Neurons with I_h</td>
<td>26</td>
<td>−75 ± 5</td>
<td>*239 ± 78</td>
<td>25</td>
<td>± 9</td>
<td>22</td>
<td>85</td>
<td></td>
</tr>
<tr>
<td>Neurons without I_h</td>
<td>34</td>
<td>−77 ± 6</td>
<td>*298 ± 102</td>
<td>7</td>
<td>± 4</td>
<td>11</td>
<td>32</td>
<td></td>
</tr>
</tbody>
</table>

Data shown as means ± SD. *Comparison of R_N between cells with and without I_h (P < 0.01).

**Heterogenous actions of ACh in non-FS interneurons**

Focal application of ACh (0.1 or 5 mM, 10–50 ms) produced a variety of pharmacologically verifiable responses in non-FS neurons in all three areas of neocortex (Tables 2 and 4). Non-FS interneurons tended to have higher input resistances than FS neurons (mean R_N = 273 ± 96 MΩ, n = 60; P < 0.0001), where compared with the R_N of FS neurons. We classified non-FS cells into two groups based on whether or not they exhibited significant depolarizing "sag" potentials (≥15%) during long hyperpolarizing current injections from rest (indicative of a hyperpolarization-activated current, I_h; Supplemental Fig. 1, Table 4).

Although the majority of non-FS cells were unresponsive to ACh (33 of 60 neurons; Fig. 9A), the percentage of nonresponding neurons was much greater in neurons exhibiting I_h (85% of cells nonresponsive to ACh) than in neurons showing little if any sag during negative current injection (32% of cells nonresponsive to ACh). Of cells responding to ACh (n = 27), 23 neurons exhibited rapid depolarizing responses to ACh that appeared clearly ionotropic in nature: mean amplitude from rest was 17.3 ± 9.7 mV, whereas mean response latency was 40 ± 18 ms and mean rise time was 78 ± 89 ms (n = 23). The reversal potential for these responses, as estimated from linear regression of the amplitude of responses at different subthreshold holding potentials, was near 0 mV, and large responses often initiated action potentials (data not shown). To confirm that these responses were due to nAChR activation, in seven neurons, the nAChR antagonist mecamylamine (10 µM) was bath-applied (Fig. 9B). In all seven cases, mecamylamine abolished depolarizing responses, indicating that they are mediated by activation of nAChRs. Nicotinic responses were observed in all cortical areas and layers. About 83% of

*The online version of this article contains supplemental data.*
found hyperpolarized non-FS neurons in all three cortical areas (n = 12), including the somatosensory cortex (n = 3), suggesting that they are present throughout the neocortex. In 11 of 16 neurons inhibited by focal ACh application, the hyperpolarization was preceded by a fast depolarizing response (Fig. 10B2) that was sensitive to mecamylamine application (10 μM; n = 4; data not shown). Hyperpolarized non-FS neurons were also similar to nicotinic responsive neurons in having very little sag potential during hyperpolarizing current steps (mean sag =

FIG. 9. Most non-FS neurons are either nonresponsive to ACh or exhibit nicotinic excitation. A1: response of a non-FS neuron from the visual cortex to positive and negative current injection (top), and the resulting I-V plot (bottom). Dashed lines indicate voltage range of linear membrane response; —, linear fit to those points. A2: recording of this neuron during the focal application of ACh (100 μM). This cell was representative of ~55% of non-FS cells tested in not showing any response to focal ACh application. B1: membrane response and voltage-current relationship for a non-FS cell from the somatosensory cortex. B2: responses to focal ACh application before and after the bath application of the nicotinic antagonist mecamylamine (10 μM). This neuron was representative of ~38% of non-FS cells in exhibiting a nicotinic response to focal ACh application. Traces in A2 and B2 are averages of 4 consecutive trials. Scale bars are the same in A1 and B1 and A2 and B2.

nAChR-responding neurons (19 of 23 cells) had only small depolarizing sag potentials during negative current injection (Supplemental Fig. 1, Table 4), confirming a negative correlation between the presence of $I_h$ and nAChR responsiveness in non-FS neurons. This difference was further reflected in the subthreshold membrane properties of these neurons, with nAChR-responding neurons having a significantly larger $R_N$ than other non-FS cells (310 ± 109 vs. 249 ± 81 MΩ, respectively; n = 60, P < 0.05).

In a much smaller fraction of non-FS neurons (4 of 60 neurons), focal ACh application (100 μM, 50 ms) generated hyperpolarizing responses (Fig. 10 and Tables 2–4). When cholinergic hyperpolarization in these neurons was challenged with atropine, responses were completely blocked (mean baseline response = −4.7 ± 1.0 mV, mean response in atropine = 0.0 ± 0.2 mV; n = 4; P < 0.01; Fig. 10B). In three neurons tested with atropine, we first applied the SK-channel antagonist apamin (100 nM). Unlike inhibitory cholinergic responses in pyramidal neurons, apamin failed to block ACh-induced hyperpolarization in any of these neurons (mean baseline response = −5.1 ± 0.7 mV, response in apamin = −5.3 ± 1.2 mV; n = 3; P = 0.72). Of our initial four neurons hyperpolarized by ACh, three were layer 2/3 neurons in the mPFC, and one was a layer 5 neuron from the visual cortex. However, in additional experiments in which large layer 2/3 neurons were specifically targeted for pharmacological and immunohistochemical experimentation (described in the following text), we

FIG. 10. A minority of non-FS neurons (~7%) showed hyperpolarizing responses to ACh. A1 and 2: subthreshold and suprathreshold membrane responses in 2 neurons showing ACh-mediated hyperpolarization. B1: superimposed responses to focal ACh application in baseline conditions (black trace) and after bath application of 100 nM apamin (gray trace). After addition of atropine (1 μM; bottom), the response disappears. Traces are averages of 4 consecutive trials in neuron shown in A1, B2: averaged response (4 consecutive trials) to ACh application to the neuron shown in A2 before (top) and after (bottom) bath application of the M2-like receptor antagonist methoctramine (MCT; 500 nM). C1: summary of responses to ACh in control conditions (n = 6) and in a subset of neurons exposed to bath-applied apamin (100 nM; n = 5) and/or atropine (1 μM; n = 4). C2: summary of pharmacology data in 2 groups of neurons (n = 6 each) that were challenged with antagonists to either M1-like (pirenzepine, PZP, 500 nM) or M2-like (MCT, 500 nM) receptor antagonists. Data shown as means ± SD.
7.9 ± 6.3%; n = 16), with the vast majority of hyperpolarized cells (15 of 16) being considered Ih-negative (Fig. 10A; Table 4). These data demonstrate that ACh can inhibit some non-FS neurons but that the mechanism mediating this inhibition is likely different from the mechanism mediating cholinergic inhibition in pyramidal neurons.

We next tested whether inhibitory responses in non-FS neurons result from M1- or M2-like receptor activation in a separate set of pharmacological experiments. For these experiments, in which immunohistochemistry was also performed (see following text), we specifically targeted the large nonpyramidal neurons in cortical layer 2/3, previously shown to be hyperpolarized during bath applications of muscarine (Kawaguchi 1997). We found 12 neurons that were hyperpolarized by focal ACh application (100 μM, 50 ms; Fig. 10, B2 and C2; Table 3). Because these neurons were specifically targeted, they are not included in Table 2. However, the amplitudes and kinetics of hyperpolarizing responses in these 12 neurons were similar to responses found in other non-FS neurons (Table 3). In baseline conditions, focal ACh application hyperpolarized neurons by 7.3 ± 2.5 mV (n = 12). After baseline measurements of cholinergic responses, six of these neurons were exposed to the M1 receptor antagonist PZP (500 nM), and the other six neurons were exposed to the M2 antagonist MCT (500 nM). PZP application reduced the amplitude of hyperpolarizing responses from a baseline value of $7.6 \pm 3.4$ to $-4.3 \pm 3.9$ mV ($P = 0.06$) but failed to abolish any responses (compare with the complete block observed in pyramidal neurons, Fig. 3, A and B). On the other hand, hyperpolarizing responses in neurons exposed to the M2-like antagonist MCT, at a concentration ineffective in blocking M1-like receptor-mediated responses in pyramidal neurons (Fig. 3, A and B), caused a significant and near complete reduction of response amplitude to $-0.9 \pm 0.6$ mV ($n = 6$; baseline response was $-6.9 \pm 1.6$ mV; $P < 0.001$; Fig. 10, B and C). These data suggest that M2-like receptors are involved in modulating the excitability of some neocortical interneurons.

**Biochemical identity of cortical interneurons**

Cortical interneurons have been classified in a variety of ways, including their differential expression of a number of biochemical markers (Cauli et al. 2000; Kawaguchi and Kubota 1997; Markram et al. 2004). Data from immunohistochemical (Kawaguchi 1997; Kawaguchi and Kondo 2002) and single-cell PCR (Porter et al. 1999) studies suggest that cholinergic responsiveness correlates with the expression patterns for VIP, CCK, SOM, and PV. To confirm these biochemical relationships, we performed additional experiments (including the pharmacology experiments for hyperpolarized non-FS cell, described in the preceding text) using thin slices of cortex (180 μm thickness) in which recorded neurons were filled with biocytin (7 mg/ml, dissolved in normal pipette saline). Thirty-six interneurons recorded in layers 2/3 and 5 were successfully labeled with biocytin and at least one additional biochemical marker. Neurons not responsive to focal ACh fell into two groups, PV-positive FS neurons (n = 6) and SOM-positive non-FS neurons (n = 5; data not shown). Biochemically identified neurons responsive to ACh application fell into three groups: CCK-positive neurons hyperpolarized via M2-like receptor activation (n = 10), CCK-positive neurons showing only nicotinic responsiveness (n = 11), and VIP-positive neurons that had nicotinic responses to ACh (n = 4, including 2 that co-expressed CCK; Fig. 11, A, B, and D). In addition, two biocytin-labeled late-spiking cells in layer 2/3 were found to have nicotinic responsiveness but were negative for both VIP and CCK (Fig. 11C). Interestingly, although similar in amplitude, latency, and rise time, nAChR-mediated responses in VIP-positive neurons were significantly longer in duration as measured at half-peak amplitude (mean = 1,300 ± 478 ms; n = 4) than were nicotinic responses in VIP-negative neurons.

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

**FIG. 11.** Neuronal responses to focal ACh application are correlated with the expression of biochemical markers. A–D: images of cells filled with biocytin and visualized after binding of Alexa-350-streptavidin (left), and immuno-staining for cholecystokinin (CCK, Alexa-594-conjugated antibody; 2nd column) and vasoactive intestinal peptide (VIP, Alexa-488-conjugated antibody; 3rd column), and the merged image (right). Far right: electrical responses showing the firing pattern to suprathreshold current injection (top) and responses to focal ACh application (bottom) for each neuron in A–D. E: summary graph showing that VIP-positive neurons have a significantly longer nAChR-response 1/2-width when compared with responses in non-VIP-positive (CCK-positive only) neurons.
neurons (mean half-width = 248 ± 119 ms; n = 10; P < 0.01; 1 CCK-positive neuron showing only suprathreshold nAChR responses was not included in this analysis; Fig. 11E). This difference in response duration was not dependent on the duration of ACh application (mean puff duration was 30 ± 16 ms in VIP-positive cells and 46 ± 31 ms in VIP-negative neurons), suggesting that it may reflect cell-type-specific differences in nAChR expression. Together, these data confirm that CCK- and VIP-positive neurons are selectively responsive to transient ACh receptor activation.

Nicotinic responses in layer 1 neurons

Finally, to confirm that ACh excites neocortical layer 1 interneurons via nAChR activation as has previously been reported (Christophe et al. 2002), we focally applied ACh to layer 1 neurons from the mPFC, somatosensory, and visual cortex (n = 5 from each area; Fig. 12). About half of layer 1 neurons were found to be late-spiking neurons (LS cells, n = 8 of 15; Fig. 12A), with the remaining neurons showing a variety of firing characteristics (Fig. 12B). No significant differences were observed in the resting potentials or input resistances of LS and non-LS neurons (mean V_M and R_S were −77.0 ± 5.3 mV and 177 ± 109 MΩ in LS cells and −77.9 ± 4.5 mV and 243 ± 140 MΩ in non-LS cells). The majority of layer 1 neurons (14 of 15) were similar to cholinergic-receptive neurons in layers 2/3 and 5 in lacking a substantial sag during prolonged hyperpolarizing current injections (mean sag = 7.2 ± 3.8%, n = 15). Responses to focal ACh application (100 μM, 50 ms) were not significantly different in LS and non-LS layer 1 neurons, and data were therefore pooled (n = 15). In all cells tested, ACh generated fast depolarizing responses from rest that appeared ionotropic in nature (mean amplitudes, latencies, and rise times were 13.0 ± 7.5 mV, 41 ± 14 ms, and 53 ± 34 ms, respectively; n = 15). Indeed, mecamylamine (10 μM, bath-applied) abolished responses in 13 of 13 neurons tested (Fig. 12, A3 and B3), confirming that these responses are mediated by nAChRs.

Together, the preceding data demonstrate heterogenous actions of ACh on non-FS neocortical interneurons, with 38% of non-FS cells in layers 2/3 and 5, and 100% of layer 1 neurons exhibiting nAChR-mediated excitation, and a minority of non-FS cells in layers 2/3 and 5 (7%) responding with an M2-like mAChR-mediated inhibition. The ability of focal ACh application to modulate CCK- and VIP-positive non-FS neurons contrasts with its inability to modulate the excitability of PV-positive FS cells and SOM-positive non-FS neurons and suggests that transient ACh release will preferentially facilitate certain inhibitory circuits, especially in the most superficial cortical layers.

DISCUSSION

The role of ACh in cortical function has been a puzzle for more than half a century with numerous papers describing excitatory and/or inhibitory effects of ACh on neocortical neurons from different animal species, from different cortical areas, and under different experimental conditions. We examined cholinergic modulation of excitability in a variety of neuron-types in several neocortical areas of the rat and report heterogenous actions of ACh that may explain some of the disparate conclusions drawn from earlier studies. Although an exhaustive investigation of phasic ACh responses in every variety of neocortical neuron is beyond the scope of this paper, our results reveal several general features of cholinergic modulation of the neocortex, including cell-type-, layer-, and region-specific effects of cholinergic receptor activation.

Cholinergic inhibition of pyramidal neurons

We found transient mACHR activation inhibits layer 5 neocortical pyramidal neurons throughout the neocortex and that cholinergic inhibition results from activation of an apamin-sensitive calcium-activated potassium conductance. In somatosensory neurons, this SK-type conductance is gated by M1-like mACHR activation and IP_3-driven increases in intracellular calcium (Gulledge and Stuart 2005). Here we confirm this mechanism also mediates cholinergic inhibition in prefrontal pyramidal neurons. Given the association of M1-like receptor activation with IP_3 generation, calcium release from internal stores, and potassium currents (Jones et al. 1988), this mechanism likely mediates inhibition in cholinoreceptive pyramidal

FIG. 12. Layer 1 neurons have nicotinic responses to focal ACh application. Representative data from both a late-spiking (LS cell, A) and a non-LS neuron (B) in layer 1. Suprathreshold (A1 and B1) and subthreshold (A2 and B2) membrane responses in these neurons. Focal application of ACh (100 μM) generated mecamylamine-sensitive nAChR responses in all layer 1 neurons (A3 and B3).
neurons throughout the neocortex. Our finding that apamin, an SK channel antagonist, blocks cholinergic inhibition of pyramidal neurons in multiple cortical areas and layers further supports this conclusion. Importantly, this mechanism is distinct from the indirect, GABA-mediated inhibition described in the guinea pig cortex (McCormick and Prince 1986). Indeed, in our experiments GABAergic antagonists did not block cholinergic inhibition, and phasic mAChR-mediated excitation of interneurons was not observed (>100 FS and non-FS cells), even after focal application of a high concentration of ACh (5 mM, n = 18) (see also Xiang et al. 1998).

Cholinergic inhibition is not a universal feature of all pyramidal neurons, however. Inhibitory responses were most prominent in deeper layers with responses being less frequent and smaller in superficial pyramidal neurons. Inhibition of layer 5 pyramidal neurons required cholinergic input near the soma, an effect that could result from differential expression of M1-like receptors or SK channels in the apical dendrites of these neurons. Although comprehensive data on the subcellular distribution of mAChRs in the intact rat neocortex is lacking (but see Wang et al. 1994), SK channels composed of SK2 subunits are restricted to the cell body and proximal dendrites of somatosensory layer 5 neurons (Sailer et al. 2002). Differential expression of mAChRs or SK channels could also explain the lack of cholinergic responsiveness in superficial neurons. Additionally, unlike deeper neurons, superficial pyramidal neurons can express the calcium-binding protein calbindin (Kawaguchi 2003), a calcium-binding protein that could interfere with the calcium-signaling necessary for ACh-mediated SK-receptor activation.

Layer 5 pyramidal neurons are themselves a heterogeneous group in terms of their axonal projections and synaptic connectivity (Morishima and Kawaguchi 2006; Wang et al. 2006), and therefore it is possible that ACh differentially gates the excitability of these neurons. However, our finding of only a single layer 5 pyramidal neuron (from the visual cortex) not inhibited by ACh suggests cholinergic inhibition is a general feature of the vast majority of layer 5 pyramidal cells, and not restricted to specific subsets of neurons. The enhanced responsiveness of layer 5 pyramidal neurons in the prefrontal cortex most likely results from subtle differences in M1-like receptor expression as can be inferred from studies showing greater densities of cholinergic axons in prefrontal cortex (Mechawar et al. 2002). Therefore it is unlikely that the larger amplitude responses in prefrontal neurons reflect a qualitative difference in cholinergic modulation of the cortex. More significant is the finding that layer 2/3 pyramidal neurons in visual cortex are more responsive than layer 2/3 neurons in other cortical areas. Although the functional role of cholinergic inhibition in pyramidal cells in layer 2/3 of visual cortex remains to be determined, our data are consistent with studies showing cholinergic-mediated (McCormick and Prince 1986; Phillis and York 1967) and SK channel-mediated (Yamada et al. 2004) inhibition in these cells, and demonstrate that phasic cholinergic inhibition is not limited to layer 5.

**Phasic cholinergic signaling in neocortical interneurons**

We found that FS neurons, previously shown nonresponsive to bath application of carbachol (Kawaguchi 1997), are also nonresponsive to transient ACh application. Inhibitory actions attributable to ACh were never observed, even when very high concentrations of ACh (5 mM) were presented to FS cells in the visual cortex of young SD rats. On the contrary, pressure application of ACh, 5-HT, or drug-free ACSF generated similar hyperpolarizing responses in ~64% of FS neurons (see Table 3). While we speculate that similar artifacts of pressure application may explain the hyperpolarizing responses to ACh reported by Xiang et al. (1998), a direct comparison between the two studies is difficult, as no quantitative description of cholinergic responses (such as amplitude, latency, rise time, or half width) was given in the previous report. Arguing against the idea that the hyperpolarizing responses in FS neurons reported by Xiang et al. (1998) are artifacts of pressure application is their observation that a cholinergic antagonist (5 mM scopolamine, drop applied) blocked responses in a limited number of cells (n = 4). However, we found no antagonistic effects of atropine and scopolamine (n = 15) at bath concentrations (1 and 100 μM, respectively) sufficient to block mAChRs. In conclusion, we believe convincing data demonstrating cholinergic modulation of FS cell excitability is still lacking.

On the other hand, a clear role for nAChR-mediated excitation of non-FS neurons has been reported by several laboratories (Christophe et al. 2002; Porter et al. 1999; Xiang et al. 1998). The biochemical and physiological profiles of nAChR-responding non-FS neurons reported here closely matches that of the nAChR-responding interneurons described by Porter et al. (1999). Additionally, our finding that layer 1 interneurons are excited via nAChRs confirms data from Christophe et al. (2002) and suggests ACh enhances inhibition within layer 1 throughout the neocortex.

Unlike earlier studies, we observed a subset of non-FS neurons (7%) that are inhibited by focal ACh application. Immunohistochemistry results confirm that these cells express CCK and suggest that they represent the large CCK-positive basket neurons previously shown to be hyperpolarized during bath application of muscarine (Kawaguchi 1997) and that make up ~5% of the total interneuron population (Y. Hirai and Y. Kawaguchi, unpublished observations). Inhibitory responses in non-FS cells were not blocked by apamin or an M1-like receptor antagonist but were sensitive to antagonism of M2-like mAChRs. Additional work will be required to determine the signal transduction pathways and ionic mechanisms of cholinergic inhibition in these relatively rare interneurons.

**Functional significance**

Our data demonstrate that transient cholinergic receptor activation has a predominantly inhibitory effect in the cortex by directly hyperpolarizing layer 5 pyramidal neurons and exciting a subpopulation of interneurons (supplemental Fig. 2). Neocortical pyramidal neurons were able to follow ACh applications up to ~0.5 Hz, rates that are several times slower than the rhythmic bursting activity of cholinergic neurons in awake animals (Jones 2004; Lee et al. 2005). Whether synthetically released ACh provides better fidelity of inhibitory responses to repetitive cholinergic input, or localized inhibition in individual dendritic branches, will require additional experiments in vivo. However, it is possible that prolonged periods of frequent cholinergic release initiate voltage-dependent excitation of neocortical pyramidal neurons (Andrade 1991; Haj-Dahmane and Andrade
1996; Knrjevic et al. 1971; McCormick and Prince 1986). With the initiation of sustained high-frequency burst-firing of cholinergic neurons, for instance, during the transition from sleep to awake (Lee et al. 2005), initial hyperpolarizing responses in layer 5 neurons might “reset” populations of output neurons to a common hyperpolarized membrane potential. After this global inhibition, voltage-dependent depolarization resulting from tonic mAChR activation could amplify the firing of neurons receiving the strongest (and hence most behaviorally appropriate) synaptic drive. Nicotinic receptor-driven inhibition via CCK- and VIP-positive interneurons, and layer 1 cells, would further sculpt both excitatory and inhibitory inputs (Christophe et al. 2002; Porter et al. 1999). In addition, rhythmic release of ACh in the cortex could dampen the firing of neurons experiencing very high levels of excitatory drive as firing frequencies increase to levels allowing spike-dependent recovery of inhibitory responses. Together these features may allow ACh to be either excitatory or inhibitory, depending on the activity state of cortical neurons, as originally suggested over half a century ago (Crossland and Mitchell 1955).

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