Cholinergic Synaptic Potentials in the Supragranular Layers of Auditory Cortex

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ABSTRACT Receptive-field plasticity within the auditory neocortex is associated with learning, memory, and acetylcholine (ACh). However, the interplay of elements involved in changing receptive-fields remains unclear. Herein, we describe a depolarizing and a hyperpolarizing potential elicited by repetitive stimulation (20-100 Hz, 0.5-2 sec) and dependent on ACh, which may be involved in modifying receptive-fields. These potentials were recorded, using whole cell techniques, in layer II/III pyramidal cells in the rat auditory cortex in vitro. Stimulation at low stimulus intensities can give rise to a hyperpolarizing response and stimulation at higher stimulus intensities can elicit a depolarizing response. The depolarizing response had a reversal potential of -35 mV, and was reduced by the combination of AMPA/kainate and NMDA glutamate receptor antagonists (AMPA/kainate: CNQX, DNQX, and GYKI 52466; NMDA: APV, MK-801) and by the muscarinic ACh receptor antagonist atropine. The hyperpolarizing response had a reversal potential of -73 mV and could be reduced by atropine, GABA_A receptor antagonists (bicuculline and a Cl^- channel blocker picrotoxin), and to a small extent a GABA_B receptor antagonist (saclofen). This suggests that the hyperpolarizing response is likely to be mediated by ACh acting on GABAergic interneurons. Extracellular recordings, also made from layer II/III of cortical slices, yielded a negative-going potential which was reduced by ionotropic glutamate receptor antagonists (same as above) and by the ACh receptor antagonists atropine and scopolamine, suggesting that this potential was the extracellular representation of the depolarizing response. Synapse 41:118-130, 2001. © 2001 Wiley-Liss, Inc.

INTRODUCTION

Neuronal receptive fields in adult neocortex can be modified in response to various learning paradigms or to peripheral injury (Rosa et al., 1995; Sachdev et al., 1998; Merzenich et al., 1996; Gilbert, 1998; Weinberger, 1998). For example, in auditory cortex neurons have frequency-receptive fields that can change as a function of pairing an acoustic stimulus (tone) with a somatosensory stimulus (foot shock) (Edeline, 1999). If the tone to be paired with foot shock does not elicit the maximal cellular response prior to pairing, then often following pairing the receptive-field profile shifts such that the paired tone elicits the maximal response and other tones, which were not paired with foot shock, elicit smaller responses. Stimulation of peripheral receptors also results in acetylcholine (ACh) release in cortex (Acquas et al., 1998; Pepeu and Blandina, 1998). Thus, it is interesting that frequency-receptive fields and rate-level functions in the adult animal can be modified by ACh acting at muscarinic receptors (Ashe et al., 1989; McKenna et al., 1989; Metherate et al.,

1990; Kilgard and Merzenich, 1998). Also, pairing a tone with iontophoretic ejection of ACh results in modification of receptive-fields such that tones paired with ACh generally elicits larger responses and unpaired tones usually elicit smaller responses (Metherate and Weinberger, 1990). Collectively, these data show that pairing tones with either somatosensory stimuli or ACh can reorganize frequency receptive fields. The mechanism underlying increased responses to significant tones and decreased or unchanged responses to other tones resulting in receptive field modification is unresolved, but studies of local circuitry and local actions of neurotransmitters may well expand understanding of the relevant mechanisms.

Studies of synaptic properties of neurons in auditory cortex have revealed some dynamic processes that may contribute to receptive field changes (Cox et al., 1994;

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Metherate and Ashe, 1994, 1995a,b; Aramakis et al., 1997a,b; Kudoh and Shibuki, 1997). Stimulation to the nucleus basalis, which has the effect of releasing ACh in the cortex (Kurosawa et al., 1988; Rasmusson et al., 1992), can change the relationship between EPSPs and IPSPs such that the net effect is to increase EPSP amplitudes and the likelihood of neuronal discharge (Metherate and Ashe, 1993). Also, when muscarinic receptors are activated by exogenous agonists, NMDAmediated responses tend to be directly enhanced, besides indirect enhancement via reduction of IPSPs (Cox et al., 1994; Aramakis et al., 1997a,b). ACh also depolarizes membrane potential and increases cellular excitability (Nishikawa et al., 1994; Metherate et al., 1992; Cox et al., 1994).

Herein we describe extracellularly and intracellularly recorded cholinergic synaptic potentials elicited by repetitive stimulation that can either increase or decrease cellular excitability. A depolarizing and a hyperpolarizing atropine-sensitive response can be elicited from the same pyramidal cell but involve different mechanisms, i.e., the depolarizing potential is apparently direct and the hyperpolarizing potential requires activation of an interneuron. Portions of this work have appeared in abstract form (Bandrowski et al., 1998).

MATERIALS AND METHODS

The auditory cortical slice preparation utilized here has been described in detail elsewhere (Cox et al., 1992, 1994; Metherate and Ashe, 1994). Briefly, male Sprague-Dawley rats (3-5 weeks old, 80-120 g) were anesthetized with Nembutal (25 mg/kg) and their brains rapidly removed and placed in ice-cold modified Krebs solution containing (in mM): NaCl 125.0, KCl 2.5, NaHCO₃ 25.0, KH₂PO₄ 1.25, MgSO₄ 1.2, CaCl₂ 2.5, glucose 10.0, and bubbled with 95% O₂, 5% CO₂. The pH of the bathing solution was approximately 7.4, and osmolarity was 298-302 mosmol/kg. Coronal slices, $350-400 \ \mu m$ in thickness, were obtained from auditory cortex (Sally and Kelly, 1988; Cox et al., 1992) with the use of a vibrating tissue slicer and placed into either an interface-type recording chamber or a holding chamber at room temperature for at least 2 h to equilibrate. Slices in the recording chamber were superfused with Krebs solution at a rate of 1.5-2 ml/min and warmed (32°C) moistened gas (95% O₂, 5% CO₂) was continuously directed over the surface.

Micropipettes for whole cell recording (WCR) and extracellular recording were made from standard glass capillary tubes. Pipettes had tip diameters of approximately 2.5 μ m (WCR) and 1 μ m (extracellular). WCR pipettes were filled with 120.0 mM K⁺ gluconate, 2 mM MgCl₂, 2 mM CaCl₂, 10 mM ethylene glycol-bis(betaaminoethylether) N, N, N', N'-tetraacetic acid (EGTA), 10 mM N-2 hydroxyethlpiperazine-N'-2 ethanesulfonic acid (HEPES), adjusted to pH 7.30 with KOH (1.0 M), and the final osmolarity was adjusted to 277–282 mosmol/kg with distilled water. DC resistances of micropipettes were 3–6 M Ω . Micropipettes for extracellular recording were filled with 3 M NaCl. Insulated stainless-steel bipolar electrodes (200 μ m tip diameter) were used for electrical stimulation and placed at a distance of 1,250–1,600 μ m from the pia, either in deep gray or underlying white matter, in line with the recording electrode. Stimulation consisted of monophasic constant current pulses (0.1 ms; 5–100 μ A).

WCR and extracellular recordings were obtained 300-450 μ m from the pia surface, corresponding to layer II/III of rat auditory cortex (Roger and Arnault, 1989). Series resistance for WCR (5–26 M Ω) was compensated using the discontinuous current clamp (switching) mode of the amplifier (Axoclamp 2A, Axon Instruments, Burlingame, CA) to measure the membrane response to steady hyperpolarizing current (0.2– 0.5 nA). Switching frequencies were 5–8 kHz (Metherate and Ashe, 1995a). Compensation was adjusted at regular intervals and series resistance generally ranged from 5–22 M Ω initially and if this value changed by more than 20% recordings were considered unreliable and were discontinued.

In some experiments (n = 14), the recording electrode contained biocytin (0.3%), so that cellular morphology could be determined. Slices were fixed in 4% paraformaldehyde for at least 24 h, resectioned at 50 μ m on a freezing microtome, and processed using the avidin-biotin-peroxidase method (ABC Kit; Vector Laboratories, Burlingame, CA; Horikawa and Armstrong, 1988; Kita and Armstrong, 1991). Neuronal morphology was reconstructed using camera lucida drawings from serial sections.

Field potentials or membrane potential (V_m) were monitored using an oscilloscope and chart recorder and computer software (AXODATA, Axon Instruments) was programmed for stimulus delivery and data collection. Recordings were digitized at a sampling rate of 5–20 kHz (MIO-16; National Instruments, Baltimore, MD), and stored on a computer (Macintosh Quadra 900).

The following pharmacological agents were applied by superfusion. Purchased from Sigma-Aldrich Co. (St. Louis, MO): atropine sulfate (Atropine), (-)-bicuculline methiodide (bicuculline), 6-cyano-7-nitroquinoxaline-2,3-dione (CNQX), picrotoxin, and scopine tropate (Scopolamine); and from Research Biochemicals Inc. (RBI, Natick, MA): R(-)-2-amino-5-phosphonovaleric acid (APV), 6,7-dinitroquinoxaline-2,3-dione (DNQX), 1-(4-Aminophenyl)-4-methyl-7,8-methylenedioxy-5H-2,3benzodiazepine hydrochloride (GYKI 52466), and (RS)-3-amino-2-(4-chlorophenyl)-2-hydroxypropylsulphonic acid (saclofen). These drugs were prepared as concentrated stock solutions in distilled water and diluted to final concentration with Krebs solution.

The magnitude of potentials recorded intracellularly or extracellularly was determined by calculating the

A. Extracellular



elicits glutamate and GABA receptor-sensitive potentials. A: Extracellular recording. Single stimulus pulses elicit a negative-going and a positive-going potential that are sensitive to glutamate receptor antagonists (CNQX 20 µM, APV 100 μ M) and GABA_A receptor antagonist (BMI 10 μ M), respectively. Repetitive stimulation at 50 Hz elicits a slow negativity (hundreds of milliseconds) in the field potential (negative shift-shaded area, EN). B: Intracellular recording. Injection of direct current (0.2 nA, 800 ms) elicits repetitive action potential firing that accommodates over the duration of the 800 ms current pulse. Inset: Camera lucida reconstruction of the pyramidal cell that gave rise to the illustrated responses: action potentials (to the left) and EPSPs (to the right). Single stimulus pulses to layer VI elicit a fast-EPSP which is blocked by CNQX 20 µM and APV 100 µM. Repetitive stimulation at 50 Hz elicits a slow (hundreds of milliseconds in duration) depolarization (slow depolarization, shaded area). Each trace is an average of 10 responses (extracellular) or 3 responses (intracellular). Calibration for the inset cell is 100 μ m.

Fig. 1. Stimulation to laver VI

area of a 1-sec period during (for most intracellular recordings) or after the repetitive stimulation (for most extracellular recordings) and therefore the units of area are mV seconds (mV*s in figures). Area was measured from the baseline immediately prior to stimulation. For intracellular recordings the area was plotted with respect to membrane potential (V_m) and these plots were used to identify reversal potentials by fitting the points to a linear regression function. Linear regression correlation coefficients (r) for all data are greater than 0.92. For extracellular analysis, changes in area are expressed as "percent change from control." Averages are presented ± 1 standard error of the mean (SEM). Differences between means were evaluated using the *t*-test for independent samples or, where appropriate, t-test for dependent samples. All differences were considered statistically significant if $P \leq 0.05$. Unless otherwise indicated, probability values in the text refer to results from *t*-tests.

RESULTS

Intracellular or extracellular recording electrodes were placed in layer II/III of rat auditory cortex (Roger and Arnault, 1989), i.e., $250-400 \mu$ m from pia. Extracellular recordings were obtained from 48 slices and intracellular recordings were obtained from 74 neurons (63 additional slices). Extracellular potentials in response to single stimulus pulses to layer VI consisted of an initial negativity followed by a positivity (Fig. 1A). The initial negativity was blocked by the combination of CNQX (20 µM) and APV (50 µM), and the positivegoing potential by bicuculline (5 μ M), as previously described (Metherate and Ashe, 1995b). For intracellular recordings (Fig. 1B) the mean resting potential was -69.5 ± 2.3 mV and input resistance (R_i) was 72.5 \pm 4.1 M Ω . R_i was measured immediately following series resistance compensation, i.e., within 5-10 min of establishing the whole-cell recording configuration. Passage of direct depolarizing current (0.2-0.4 nA, 800 ms) elicited repetitive spike discharge which accommodates during the 800 ms depolarizing pulse; a characteristic associated with pyramidal cell physiology (Connors et al., 1982; McCormick et al., 1985; Connors and Gutnick, 1990). In addition, 14 cells were filled with biocytin and identified as having pyramidal cell morphology (White, 1989; Nieuwenhuys, 1994; Fig. 1). Monophasic stimulus pulses delivered to layer VI elicited fast and slow synaptic potentials, i.e., fast-EPSP (Fig. 1B) and slow-EPSP (CNQX and APV sensitive, respectively) and fast-IPSP and slow-IPSP (bicuculline and saclofen sensitive, respectively), as described previously (Cox et al., 1992; Metherate and Ashe, 1994; Aramakis et al., 1997b).

Repetitive stimulation: Characteristics of extraand intracellular responses Extracellular responses

Extracellular responses were elicited by repetitive stimulation at frequencies ranging from 2–100 Hz, durations ranging from 0.1–2 sec and at intensities rang-

120



Fig. 2. Repetitive stimulation to deep gray elicits a slow, extracellularly recorded negative-going response. A: Increasing stimulus strength increases the extracellular negativity. Stimulus strength was increased by increasing one of three parameters: stimulus frequency (5–100 Hz), stimulus duration (0.1–2 sec), or stimulus current (7.5–45 μ A). Increasing any of these parameters produces a negativegoing response that is larger in amplitude and longer in duration. B: Examples of high (50 Hz) and low (20 Hz) strength trains from different slices. i. Stimulation at 20 Hz (1 sec) elicits an extracellular negativity (EN), with a return to baseline (0–3.8 sec) that fit an

ing from 5–100 μ A. The response elicited by these stimulus trains consisted of an extracellular negativity (EN; Figs. 1A, 2) both during and after the train (48/48 slices). Increasing stimulus strength by increasing frequency, duration, or intensity produced larger amplitude and duration negativities (22/22 slices; Fig. 2A) and appeared to result in recruitment of an additional potential (Fig. 2B). Figure 2B shows that when stimulus strength is relatively low, i.e., 20 Hz (1 sec), a negativity is elicited which peaks in amplitude at the end of the train and exponentially returns to baseline (Fig. 2Bi; 18/22 slices). In contrast, when stimulus strength is increased, i.e., 50 Hz (1 sec) a negativity that peaks after the train is elicited; however, this

exponential function ($\tau_1 = 0.801$, $\tau_2 = 1.145$). Shading indicates the response area of the EN measured (see text). **ii**. Stimulation at 50 Hz (1 sec) also elicits an EN with a return to baseline (0-3.8 sec) that fit an exponential function ($\tau = 2.35$). However, the exponential fit fails to take into account the initial 1 sec after the train. Three separate exponential equations are required to fit the fast decreasing phase (0-0.15 sec; $\tau = 0.083$), the slower increasing phase (0.2-0.9 sec; $\tau = 0.432$), and the slowest decreasing phase (1.4-3.8 sec; $\tau = 2.562$), which suggests that several overlapping processes are involved. Each trace is an average of 10 responses.

negativity cannot be fitted to a single exponential decay function (Fig. 2Bii).

Because the responses to 50 Hz (1 sec) trains were typically very robust and the negativity was likely the result of more than one component (Figs. 2B, 4A), we used these parameters as a standard for the remainder of the study. Responses did not significantly change over 1 h when stimulation was given at 1–2 min intervals (12/12 slices). However, if trains were delivered at the rate of 1/30 sec or less, the negativity becomes progressively smaller (4/4 slices). The total duration of the negativity was typically about 4 sec (after the train offset); however, it could last up to 7 sec. For purposes of analysis, unless otherwise specified, the area of the



Intracellular

122

Fig. 3. Low and high stimulus intensities elicit two intracellular responses, depolarizing (DP) and hyperpolarizing (HP). A: Both responses (HP and DP) are recorded from the same cell. Low SI: At 5 μ A, the response to stimulation (50 Hz, 1 s) reverses polarity between -75 and -85 mV. High SI: At 10 μ A, the response to stimulation (50 Hz, 1 s) is largest in area at -95 mV, and smallest at -65 mV. Each trace is an average of 3 responses. Shading indicates location of area measurement. B: Graphic representation of the data in A. The area encompassed by each potential (HP or DP) is measured for 1 sec (shaded area in A, measured in units of mV multiplied by seconds) and plotted with respect to membrane potential (V_m). Because the area after stimulation, the area during the train was used in analysis of

extracellular negativity was measured for 1 sec after the train offset to take into account amplitude and duration parameters (shaded area, Fig. 2B).

Intracellular responses

Repetitive stimulation at frequencies between 20 and 100 Hz (1–2 sec) elicited *either* a depolarizing (slow-DP; n = 26; Fig. 1, 3A) or hyperpolarizing potential (slow-HP; n = 15; Fig. 3A). Both potentials typically lasted longer than the duration of the stimulus trains, i.e., 1–4 sec. Whether a depolarizing or hyperpolarizing response was elicited depended on stimulus intensity, i.e., in the same cell lower stimulus intensity resulted in a predominantly hyperpolarizing response, and higher intensities resulted in a predominantly depolarizing response (n = 1000

intracellular responses. The variance following stimulation may indicate that several different processes are involved in determining response polarity after stimulation. Best fit lines were determined by linear regression analyses. The best fit line intersects the X-axis, and the response reverses polarity at -82 mV (HP) and more positive than -60 mV (DP). C: Graphic representation of the responses elicited in five cells, by low SI (HP) and high SI (DP). Best fit lines were determined by regression analysis. The X-intercept for each line (reversal potential) was -71 mV (HP) and -42 mV (DP), which is similar to the reversal potential values arrived at by taking the average of individual cells reversal potentials, i.e., -73.8 mV (HP; n = 15) and -35.1 mV (DP; n = 26; see text).

9). The area encompassing these responses was plotted with respect to membrane potential (V_m) and fit to a linear function which was used to determine reversal potentials (Fig. 3B). The reversal for the hyperpolarizing response was -73.8 ± 2.8 mV (15/15; Fig. 3C), whereas the depolarizing response reversed at -35.1 ± 6.9 mV (26/26; Fig. 3C). However, the depolarizing potential often reached spike threshold before reversing in polarity. This obscured an accurate measurement of the reversal potential and the indicated value represents an extrapolation.

Both the depolarizing and hyperpolarizing responses were capable of influencing cellular excitability. Responses to a single stimulus, subthreshold for spiking, could reach spike threshold when elicited in the presence



Fig. 4. An extracellular negativity and an intracellular depolarization are reduced by ionotropic glutamate receptor (iGluR) antagonists. A: Extracellular recording. Stimulation at 50 Hz (1 sec) elicits an extracellular negativity that is sensitive to iGluR antagonists. Superfusion of CNQX (20 µM) and APV (100 μ M) results in a 36% reduction of the negativity. Note that the decay of the predrug response is most likely composed of several overlying processes (see deflection at arrow), but after iGluR application the deflection is absent. Each trace is an average of 10 responses. Graph: In the presence of iGluR antagonists the area of the negativity is reduced by $53.1\,\pm\,6.4\%$ of predrug values on average (n = 8). **B:** Intracellular recording. i. Stimulation, 50 Hz (1 s., 20 µA), elicits a depolarizing response that is reduced by iGluR antagonists (DNQX 40 µM, MK-801 40 µM, and GYKI 52466 20 μM). The effect is greater during stimulation (open circles) than after stimulation (open squares). Each trace is an average of three responses. ii. Measurements taken during stimulation indicate that the slope of the best fit line is reduced from 0.33-0.07 (area/mV), a reduction of 79%. The reversal potential is 15 mV more negative in the presence of iGluR antagonists, bringing the reversal into the range of hyperpolarizing response reversal potentials (-66 mV). iii. Measurements taken after stimulation indicate that the slope of the best fit line is reduced from 0.12-0.03 (area/mV), a 75% reduction. The reversal potential was not as negative as the predrug reversal, suggesting that in this cell the two phases (during and after stimulation) reflect different processes.

of the depolarizing response. Under some conditions the depolarizing response itself generated spiking (11/26 cells), indicating that the response was excitatory. Spiking was reduced during the hyperpolarizing response, indicating that it is inhibitory in nature. In 5 of 5 cells a brief depolarizing current that elicited 2–3 spikes produced fewer spikes during the hyperpolarizing response.

Glutamate mediates a portion of the extracellular negativity and depolarizing response

Several ionotropic glutamate receptor (iGluR) antagonists were used to reduce the influence of AMPA/ kainate and NMDA receptors. AMPA/kainate antagonists (CNQX 20–60 μ M or DNQX 20–60 μ M, and GYKI 52466 20–40 μ M) and NMDA antagonists (APV 50–100 μ M or MK-801 20–40 μ M) were used in combination so that both types of receptors were blocked. This combination of antagonists completely blocked the response to single stimulus pulses (Fig. 1) and reduced the slow negative-going response to stimulus trains (Fig. 4A). Incremental increases of antagonist concentrations were examined in two slices, i.e., CNQX and MK-801 were both increased from 20–40 μ M, but the slow-extracellular negativity was not reduced further, indicating that 20- μ M concentrations produced a maximal or near-maximal block of glutamate receptors. Thus, data obtained at all concentrations of iGluR an-



Extracellular

Fig. 5. Atropine reduces the extracellular negativity. A: Stimulation (50 Hz, 1 sec) elicits an atropine sensitive negative-going response. Atropine (10 μ M) reduces the response area by 44% of the predrug area. Note that the pre-atropine return of membrane potential to baseline appears to occur in three distinct phases: decreasing, increasing, and decreasing again; however, in atropine the return to baseline is reduced to two phases. B: In the presence of iGluR antagonists, atropine reduces the amplitude and duration of the extracel-

tagonists were grouped together. The result of these manipulations was that the slow-extracellular negativity was reduced, but not eliminated, i.e., the average measured response area after the train (mV*s) was reduced from 1.183 to 0.673, a 56.9% reduction (n = 8; Fig. 4A).

The intracellularly recorded slow-depolarization, like the extracellularly recorded slow-negativity, was reduced but not eliminated even at the highest concentrations of iGluR antagonists used (n = 11; Figs. 4B,7C; AMPA/kainate and NMDA receptor antagonists and concentrations used are the same as for extracellular recordings). To quantify this effect, we calculated the area *after* the train and found that the slope of the best fit line was reduced on average by $46.8 \pm 12.3\%$ in the presence of iGluR antagonists (Fig. 4B, O). However, the portion of the depolarizing response, which occurred *during* the train, was more sensitive to these antagonists than the portion after the train. In seven of 11 cells the slow-depolarizing response could not be observed *during* the train when iGluR antagonists were present; rather, a hyperpolarizing response was noted. In these cases, the reversal potential for the response was 15–37 mV more negative than the predrug response (for example, see Figs. 4Bii, 7Bii). The reversal of the slow-hyperpolarizing response, in the presence of iGluR antagonists (-72.8 ± 4.7 mV), was not different from the reversal potential noted in the absence of antagonists $(-73.8 \pm 2.8 \text{ mV}, P > 0.05)$, suggesting that these two responses reflect the same mechanism. In the remaining four of 11 cells the depolarizing response was reduced in amplitude, but a hy-

lular negativity. The negativity, elicited by 50 Hz (1 sec) stimulation, in the presence of CNQX 20 μ M and MK-801 20 μ M is reduced by atropine (10 μ M) by 42%. All traces are averages of 10 responses. C: Plot of the area encompassed by the extracellular negativity. Mean percent of control values (\pm SEM) represent the response area in atropine divided by the preatropine response area (either predrug or iGluR antagonists).

perpolarizing potential was not completely revealed, i.e., the slope was decreased but the reversal potential was not shifted in the hyperpolarizing direction.

Cells in which the hyperpolarizing response was predominant, such as in Figure 7B (Low SI; n = 6), there was relatively little change in the presence of glutamate receptor antagonists. Neither the slope of the best fit line nor the reversal potential of the hyperpolarizing response was significantly changed in the presence of iGluR antagonists (P > 0.05; Fig. 7B, Low SI). The reversal potential usually remained within 5 mV of the original reversal potential.

These data suggest that the depolarizing response is, in part, mediated by glutamate acting at AMPA/kainate and NMDA receptors. On the other hand, the hyperpolarizing response is not mediated by activation of these receptors, indicating that the hyperpolarizing response is distinct from auditory cortical, single pulseelicited, disynaptic IPSPs, which are blocked by iGluR antagonists (Cox et al., 1992).

ACh mediates a portion of the extracellular and intracellular responses

Muscarinic receptor antagonists, atropine or scopolamine (5–10 μ M), reduced the extracellular negativity by 42% (atropine n = 7; Fig. 5A,C) or 50% (scopolamine n = 4). The effect of atropine occurred in the presence of glutamate receptor antagonists (CNQX and MK-801 or APV; n = 4; Fig. 5B,C), suggesting that atropine is not acting via iGluRs to produce its effects.

Unlike the extracellular negativity, the area encompassed by the intracellular slow-depolarizing response



Fig. 6. Atropine reduces intracellularly recorded potentials. A: Atropine differentially effects the depolarizing response. Stimulation (50 Hz, 1 sec, 50 μ A) of deep gray matter elicits a depolarizing response at -65 mV (shaded area), which is reduced by atropine (10 μ M, bottom traces). At -45 mV the predrug response includes some hyperpolarizing components both during and after stimulation. At -45 mV atropine increases the area of the depolarizing response and no hyperpolarizing components are present, suggesting that atropine may be acting to reduce the hyperpolarizing components in addition to depolarizing components. Traces are averages of three responses. Graph: Representation of the response area of the depolarizing response over the range of -45 to -75 mV. Note that at more hyperpolarized V_m (i.e., more negative than -56 mV) atropine reduces the

was larger or smaller in the presence of atropine (5–10 μ M; 8/11). Of the eight cells where atropine modified the area encompassed by the response, six responses were decreased and two were increased. We noted that the decrease occurred in cells where the resting V_m was relatively more negative (-62 to -80 mV) than those cells where the response increased, i.e., V_m more positive than -62 mV. To test whether V_m was an important factor in determining the direction of change due to atropine, V_m was systematically altered by injection of direct current (n = 3). Figure 6A shows that with a

area of the DP, but at depolarized V_m , -56 mV atropine increases the area. The slope of the best fit line is reduced by 88.6% but the reversal potential shifts in the positive direction, suggesting that multiple components are effected by atropine. B: The hyperpolarizing response is decreased in atropine. Stimulation of deep gray matter (50 Hz, 1 sec, 20 μ A) in the presence of CNQX (20 μ M) and MK-801 (20 μ M) elicits a hyperpolarizing response, which reverses polarity at -71 mV. The response at both -50 and -80 mV is reduced by atropine (10 μ M). Graph: Representation of data on left. The slope of the best fit line is reduced by 58.4% by atropine reflecting the decrease in response size. The reversal potential, pre-atropine, is -71 mV and is unaltered by application of atropine, suggesting that a single potential is being effected. Traces are averages of two responses.

 V_m of -65 mV or more, the depolarizing response decreased in area with exposure to atropine, but at V_m less negative than -65 mV, i.e., -45 mV, the response increased, indicating that the effect of atropine differs with V_m . Reflecting this change, the reversal potential of the slow-depolarizing response shifted more than 30 mV in the positive direction (n = 3) and the slope of the best fit line was reduced by 67.4 \pm 10.2% (n = 3).

To account for the large change in reversal potential, we suggest that atropine was not only reducing the depolarizing response, but also the hyperpolarizing response and that these responses temporally overlap. Both depolarizing and hyperpolarizing responses are often elicited simultaneously, as portions of the total response elicited are often both depolarizing and hyperpolarizing (33/41 cells, see Figs. 3A, 4B, 6A, 7B). Therefore, if atropine is decreasing hyperpolarizing components of the composite response at V_m more positive than the reversal of the hyperpolarizing response the composite response should increase. Thus, we sought to determine the effect of atropine on the hyperpolarizing response.

The hyperpolarizing response (relatively isolated by stimulating with low current intensity) was reduced in atropine (10 μ M; n = 5). Figure 6B shows that this effect was also observed in the presence of glutamate receptor antagonists, which blocked a portion of the depolarizing response (n = 3). Furthermore, the magnitude of the reduction was the same regardless of whether V_m was depolarized or hyperpolarized (Fig. 6B, graph). The slope of the best fit line was reduced by $52.4 \pm 5.2\%$ (n = 8) with a tropine application, without a shift in the reversal potential, suggesting that atropine decreased the magnitude of a response with a reversal potential of roughly -70 mV. This observation is consistent with the hypothesis that the hyperpolarizing response is atropine-sensitive, and that the increase in the depolarizing response may be a result of disinhibition.

Given that the hyperpolarizing response is sensitive to atropine at V_m 's more negative than -70 mV, the depolarizing and hyperpolarizing responses are both depolarizing and reduction of either response would have the effect of reducing the composite potential. Therefore, we pharmacologically isolated the depolarizing response by blocking inhibitory conductances with either picrotoxin (10 μ M; n = 3; Fig. 7B), or bicuculline (10 μ M; n = 4), and in several cells saclofen (n = 4). These manipulations were effective in reducing the hyperpolarizing response (Fig. 7B,C). Under these conditions, i.e., in the presence of picrotoxin and glutamate receptor antagonists, a depolarizing response was elicited in two of seven cells. In the presence of iGluR antagonists, saclofen, and bicuculline, a depolarizing response was elicited in two of three cells. The depolarizing response was reduced by atropine in all four cells in which it was elicited (Fig. 7C). Atropine reduced the slope of the best fit line for the depolarizing response by an average of $48.9 \pm 6.8\%$. This reduction in the slope occurred with *no* accompanying change of reversal potential, suggesting that a single potential is being reduced by atropine. These data also indicate that atropine reduces the amplitude of the depolarizing response independent of reducing the hyperpolarizing response. Also, consistent with these observations are data indicating that the extracellular negativity (which is likely the counterpart of the intracellular depolarizing response) was enhanced

in picrotoxin (8/8 slices), and subsequently reduced in atropine (6/6; Fig. 7A). These findings suggest that the hyperpolarizing response is most likely the result of a $GABA_A$ -receptor mediated Cl^- conductance and that the depolarizing and hyperpolarizing responses are, at least partly, mediated by activation of muscarinic ACh receptors.

DISCUSSION AND CONCLUSIONS

The major findings of this study can be summarized as follows. 1) ACh can give rise to both a depolarizing and a hyperpolarizing response, depending on the strength of repetitive stimulation. 2) The depolarizing response is excitatory and elicited by direct activation of pyramidal cells. 3) The hyperpolarizing response is inhibitory and mediated by ACh driving GABAergic interneurons.

A large body of evidence supports the view that ACh is present and plays an important modulatory role in neocortex. ACh-containing fibers are found (Hutsler and Gazzaniga, 1996; Woolf, 1991) and form synapses with dendritic shafts, spines, and to a smaller extent cell bodies of pyramidal cells and other cell types in neocortex (Mrzljak et al., 1995). Physiological evidence indicates that ACh or its analogs can depolarize cells and modify membrane excitability parameters (Felder, 1995). Also, there is a wealth of data indicating an involvement of ACh in modification of sensory cortical responses and receptive fields (Edeline, 1999; Pepeu and Blandina, 1998). The vast majority of these studies have implicated muscarinic receptors. Because they are atropine sensitive, responses recorded in the present study also appear to require muscarinic receptors. This is of particular interest because muscarinic actions of ACh have been shown to modify, in significant ways, glutamatergic and GABAergic synaptic potentials. These modifications are robust, long-lasting, and appear to require adjustment to intracellular biochemical state (Cox et al., 1994; Aramakis et al., 1997; Metherate et al., 1992; Metherate and Ashe, 1993, 1995b; Kimura and Baughman, 1997; Maalouf et al., 1998; Wang and McCormick, 1993; Wang, 1999; Xiang et al., 1998; Kawaguchi, 1997). The work of identification of atropine-sensitive synaptic drive onto auditory cortical neurons, and the conditions necessary for those responses, i.e., stimulus strength profiles, possible loci, and polarity of ACh's effects, provides the basis for future functional studies for ACh's role in sensory cortical function. One important and immediate question that remains concerns the receptor subtypes that are involved in the multifaceted effects of ACh released by repetitive stimulation. Any or all of the five identified receptor subtypes (Brann et al., 1993; Levey, 1993) may be involved. It may be that the M1, M3, or both, receptor subtypes are involved, because these have been associated with increases in PI metabolism (Peralta et al., 1988; Felder, 1995), decreases in various K⁺



Membrane Potential (mV)

Fig. 7. The extracellular negativity and intracellular depolarizing response are sensitive to atropine in the presence of picrotoxin. A: Extracellular recording. Stimulation (50 Hz, 1 s, 20 µA) of layer VI elicits an extracellular negativity, which is larger in the presence of picrotoxin (10 μ M). Subsequent addition of atropine (10 μ M) reduces the area from that recorded in picrotoxin. The response returns to predrug levels, with washout of picrotoxin and atropine. Graph: Averages of the effect of picrotoxin and atropine presented in graphic form (n = 9). **B:** Intracellular recording. The hyperpolarizing response is sensitive to picrotoxin. i. Stimulation at 30 μ A (High SI, 50 Hz, 1 sec) of deep gray matter elicits a depolarizing response. In the presence of iGluR antagonists (CNQX 20 µM and MK-801 40 µM) a hyperpolarizing response is revealed. Picrotoxin (10 µM) reduces the response, but does not block it. ii. Graph representing data from cell in Bi. Predrug responses reverse polarity at -55 mV (open circles). Addition of iGluR antagonists result in a response that reverses potential at -79 mV (closed circles) and picrotoxin further shifts the reversal potential to -86 mV (closed squares). iii. Stimulation of the same cell (as i) at 20 µA (Low SI, 50 Hz, 1 sec) produces a hyperpolarizing response, which is unaltered in the presence of iGluR antag-

onists, but is blocked by picrotoxin. iv. Graph representing data from iii. The predrug response (open circles) had a reversal potential of -72 mV and iGluR antagonists (closed circles) did not change the reversal potential (-74 mV). However, subsequent application of picrotoxin blocks the HP. Traces are averages of two responses. C: The depolarizing response is reduced by atropine. 1) In the presence of iGluR antagonists (CNQX 20 µM, APV 100 µM) stimulation (40 µA) elicits a hyperpolarizing response. 2) The hyperpolarizing response is reduced with the application of saclofen 100 μ M. 3) Application of BMI (10 µM) reveals a depolarizing response. 4) Atropine (10 µM) reduces the depolarizing response. Note that the response is reduced equally during and after stimulation. Graph: Plot of data on left. In the presence of iGluR antagonists a hyperpolarizing potential is elicited (open circles) and this potential is partially sensitive to saclofen (closed circles). Note the slope is reduced and the reversal potential is unchanged. Further addition of BMI shifts the reversal potential about 30 mV positive, revealing a depolarizing potential, which is sensitive to atropine. Note application of atropine reduces the slope, but not the extrapolated reversal potential, suggesting that atropine is acting to reduce a single component.

conductances (Cole and Nicoll, 1984; Benardo and Prince, 1982; Krnjevic et al., 1971), and activation of these receptors has been shown to result in membrane depolarization (Felder, 1995). Additionally, the muscarinic potential recorded from layer V neurons of auditory cortex is sensitive to the M1/M3 selective antagonist pirenzepine, but not the M2 selective antagonist AF-DX 116 (Cox et al., 1994). Thus, it seems likely that the M1 and/or M3 muscarinic receptor subtypes are involved in the production of the atropine-sensitive responses.

Depolarizing potential

Our data indicate that muscarinic as well as AMPA/ kainate and NMDA receptors antagonists were necessary to maximally reduce the depolarization. However, in the presence of both glutamate and muscarinic antagonists a portion of the depolarizing potential remains (about 15%), indicating that another component, as yet unidentified, may be present within the composite response. This remaining portion may be the result of noradrenergic (Benardo, 1993), nicotinic (Xiang et al., 1998), metabotropic glutamate receptor-mediated responses (Eaton and Salt, 1996; McCormick and von Krosigk, 1992), or other synaptic events.

The generation of the depolarizing response can occur in at least two ways: 1) by directly activating muscarinic receptors on pyramidal cells, or 2) by activating muscarinic receptors on excitatory interneurons which then release glutamate and depolarize the pyramidal neurons. Early experiments, which have been repeated in multiple systems, have shown that ACh application can excite and depolarize cells directly, including pyramidal cells within the neocortex (Krnjevic, 1975). Furthermore, recent evidence shows that interneurons of various types can also be depolarized by ACh application (Kawaguchi, 1997; Xiang et al., 1998). The present data suggest that the muscarinic portion of the depolarizing response does not occur by activation of muscarinic receptors on excitatory interneurons, because in the presence or absence of iGluR antagonists the depolarizing response (and extracellular negativity) was reduced by atropine. Therefore, it may be that the muscarinic portion of the depolarizing response is direct onto pyramidal cells.

The response recorded here from layer II/III has different pharmacological sensitivity from the atropine-sensitive slow-EPSP elicited from layer V, which was not reduced by iGluR antagonists (Benardo, 1993). This difference may be the result of the anatomical distribution of ChAT positive fibers, i.e., more AChcontaining fibers are present in layer V than layers II/III (Lysakowski et al., 1986; Descarries et al., 1997). Stimulation may thus produce smaller cholinergic responses in layers II/III than V. Additionally, different receptor subtypes distribution between layers II/III and V could have some influence on relative response size. Levey et al. (1991) reported that the M4 receptor subtype was highly localized to the neuropil of layer II/III and that the M2 subtype was more predominant in layer IV. If so, the actions of ACh in layer II/III may differ from its actions in layer V. Thus, distribution of cholinergic fibers and receptors may be an important factor for understanding the role of cholinergic modulation of neocortical microcircuits. It may be illuminating to determine if there are clear physiological differences associated with anatomical differences between layers II/III and V, as suggested by Lysakowski et al. (1986). Methodological differences between the present report and previous work by Benardo (1993) may also account for the differences in results. The apparent insensitivity to iGluR antagonists may be a result of the simultaneous application of both iGluR antagonists and picrotoxin. In our results picrotoxin increases the negative-going response by 180%, and iGluR antagonists reduce the response by 50%, suggesting that if both were applied together the composite response would increase, as described by Benardo (1993).

Hyperpolarizing potential

The hyperpolarizing response is reduced by atropine, saclofen (GABA_B antagonist), and either bicuculline or picrotoxin (GABA_A receptor antagonist, and the Cl⁻ channel blocker, respectively; Chebib and Johnston, 1999), suggesting that both muscarinic and GABA receptors are involved in initiating the response. We further suggest that ACh stimulates GABA-releasing interneurons and that GABA released from these interneurons activates both GABA_A and GABA_B receptors. For many years now it has been established that iontophoretic application of ACh in vivo results in both neuronal excitation and inhibition (Krnjevic, 1975). Similarly, in vitro application of ACh has been shown to hyperpolarize and depolarize pyramidal neurons (McCormick and Prince, 1985, 1986). The depolarization was shown to be a direct effect of turning off the M-current and the hyperpolarization was concluded to be due to an excitation of interneurons, as it was sensitive to block by $\ensuremath{\mathsf{GABA}}_A$ antagonists and TTX. More recently, Nicoll et al. (1996), Kawaguchi (1997), and Xiang et al. (1998) showed that several types of inhibitory interneurons can be depolarized by ACh and suggested that activation by ACh can give rise to inhibitory responses in pyramidal cells. Ultrastructural evidence supports this assessment, in that ChAT-positive fibers made synapses with GABAergic-like cells (Mrzljak et al., 1995), ACh receptors were found on somata, dendrites, and axons of identified neocortical and hippocampal interneurons (Csillik et al., 1998; Hajos et al., 1998), and ample evidence exists for interneuron to pyramidal cell synapses (Thomson and Deuchars, 1997). The present data adds to the established data the evidence that ACh released from cholinergic terminals activates GABAergic interneurons and that these subsequently release GABA, producing the hyperpolarizing response. The lower threshold for the hyperpolarizing response than the depolarizing response may be the result of ACh activating interneurons more powerfully than pyramidal cells, because interneurons tend to have higher input resistances than pyramidal cells (McCormick et al., 1985). Thus, the same amount of current might have larger effects on interneurons compared to pyramidal cells.

The slow-hyperpolarizing response recorded here is unlike GABAergic IPSPs previously described, because iGluR antagonists, which block IPSPs in auditory cortex (Cox et al., 1992; Aramakis et al., 1997b), do not affect the amplitude of the hyperpolarizing response. Furthermore, the hyperpolarizing response is elicited at a lower stimulus intensity than the depolarizing response (typically 5–25 μ A), again in contrast to disynaptic IPSPs which are activated by higher stimulus intensity than EPSPs. These data suggest that the hyperpolarizing response is not elicited through the same pathway as the single stimulus elicited IPSPs, i.e., by glutamate acting on interneurons which in turn release GABA.

Benardo (1993) described a slow-IPSP in layer V cells elicited by repetitive stimulation. This response seems to also be different than the presently described hyperpolarizing response because the layer V potential was elicited following spike discharge, was insensitive to picrotoxin or atropine, and reversed polarity at -85mV. The hyperpolarizing response described here is sensitive to picrotoxin and atropine, reverses polarity at -70 mV, and occurs in the absence of action potential firing. The difference in hyperpolarizing potentials between layer II/III and V may have an anatomical basis. Layer V interneurons which project within layer V have been found to be inhibited by ACh, but layer V interneurons which project to supragranular layers are activated by ACh (Xiang et al., 1998). These data suggest that cholinergic hyperpolarizing responses would be less likely to occur in layer V.

Implications for modification of receptive fields

Because ACh is present in the neocortex at a low level (Acquas et al., 1998; Kurosawa et al., 1988; Pepeu and Blandina, 1998; Rasmusson et al., 1992), and because increasing that level by iontophoretic application of ACh, or behavioral testing, induces a stimulus specific modification of cellular receptive fields (Acquas et al., 1998; Kilgard and Merzenich, 1998; Metherate and Weinberger, 1990), we and others suggest that the level of ACh, and perhaps other neuromodulators, are of paramount importance in processing sensory information and gating plasticity (Centonze et al., 1999; Edeline, 1999; Weinberger, 1998; Wang, 1999; Dickson and Alonso, 1997). The present data show that lower levels of cholinergic fiber activation, and presumably lower levels of ACh release, result in hyperpolarization and higher levels of ACh release result in a depolarization. Furthermore, the hyperpolarizing potential is inhibitory and the depolarizing potential is excitatory. Thus, if an auditory volley associated with a tone was accompanied by depolarization or hyperpolarization, then the response to that volley should increase or decrease, respectively. These inverse changes in response size may be key to shifting the best frequency of a receptive field, in that receptive field shifts necessarily require a differential treatment of significant and insignificant stimuli. This task can be accomplished in at least three ways: increase responses to important stimuli, decrease responses to unimportant stimuli, or both. The data show that cholinergic depolarizing and hyperpolarizing potentials can be readily elicited in the same cell, depending on the strength of input, suggesting that, via elicitation of these cholinergic potentials, pyramidal cell responses to some stimuli may increase and to others decrease.

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