Endogenous Acetylcholine Enhances Synchronized Internuron Activity in Rat Neocortex

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Bandyopadhyay, Susanta, Bernd Sutor, and John J. Hablitz. Endogenous acetylcholine enhances synchronized internuron activity in rat neocortex. J Neurophysiol 95: 1908–1916, 2006. First published December 7, 2005; doi:10.1152/jn.00881.2005. Application of 4-aminopyridine (4-AP) along with EAA receptor antagonists produces γ-aminobutyric acid (GABA) receptor-dependent synchronized activity in interneurons. This results in waves of activity propagating through upper cortical layers. Because interneurons in the neocortex are excited by nicotinic acetylcholine receptor (nAChR) agonists, ACh may influence synchronization of these local neocortical interneuronal networks. To study this possibility, we have used voltage-sensitive dye imaging using the fluorescent dye RH 414 (30 μM) in rat neocortical slices. Recordings were obtained in the presence of 4-AP (100 μM) and the EAA receptor antagonists 6-cyano-7-nitro-quinoxaline-2,3-dione (10 μM). Application of the ACh esterase inhibitor neostigmine (10 μM) increased the response amplitude, the extent of spread, and the duration of this activity. These changes were seen in 13 of 16 slices tested with neostigmine (10 μM) and 4 of 7 slices tested with DMPP (10 μM). Application of the muscarinic ACh antagonist atropine (1 μM) did not block the enhancement of activity by neostigmine (n = 7). Application of dihydro-β-erythroidine (10 μM), known at this concentration, to selectively antagonize α4β2-like nAChRs, blocked the effect of neostigmine (n = 5). The selective α7-like nAChR antagonist methyllycaconitine (50 nM) was ineffective (n = 5). These results suggest that activation of α4β2-like nAChRs by endogenously released ACh can enhance synchronized activity in local neocortical inhibitory networks.

INTRODUCTION

The neocortex receives a major cholinergic innervation via cholinergic afferents from the nucleus basalis of Meynert (Mesulam et al. 1983). ACh acts via two different types of receptors: muscarinic and nicotinic. Muscarinic receptors are metabotropic and thought to predominantly mediate postsynaptic effects of ACh in the CNS (McCormick and Prince 1986), although evidence exists for a presynaptic function (Sheridan and Sutor 1990). Nicotinic ACh receptors (nAChRs) are ligand-gated ion channels that mediate fast excitatory synaptic transmission in the peripheral nervous system. Presence of nAChRs has been detected in different brain regions including the neocortex. Both homo- and heteromeric receptors exist in the brain (Le Novere et al. 2002; Sargent 1993; Wada et al. 1989). Heteromeric α4β2 and homomeric α7 nAChRs are the most abundant and widely expressed nAChRs (Wada et al. 1989). nAChRs in the CNS are important for neuronal development, attention, learning and memory, nociception, and cognition (for review, see: Jones et al. 1999; Levin 2002; Picciotto et al. 2000; Role and Berg 1996). Neuronal nAChRs play a role in the pathogenesis of brain disorders such as epilepsy, schizophrenia, Alzheimer’s disease, and Parkinson’s disease (for review, see: Lena and Changeux 1997; Lucas-Meunier et al. 2003; Newhouse et al. 1997; Raggenbass and Bertrand 2002). Linking human autosomal-dominant nocturnal frontal lobe epilepsy (ADNFLE) to a mutation in the nACh α4 subunit (Steinlein et al. 1995, 1997) underscores the importance of nAChRs in modulation of circuit excitability in the neocortex. Pre- and postsynaptic modulation of glutamate release by nAChR has been demonstrated in the olfactory bulb (Alkondon et al. 1996), the hippocampus (Gray et al. 1996; Radelcliffe and Dani 1998), and the prefrontal cortex (Gioanni et al. 1999). Increases in the release of dopamine, norepinephrine, and serotonin in response to nicotinic stimulation in prefrontal cortex have also been reported (Rao et al. 2003). Studies in rat hippocampal and human neocortical slices suggest that nAChRs can trigger γ-aminobutyric acid (GABA) release from interneurons as well (Albuquerque et al. 2000; Alkondon et al. 1997, 1999). Glutamatergic neurotransmission is enhanced by nicotinic agonists in the nucleus accumbens, whereas muscarinic agonists have an opposite effect (Zhang and Warren 2002). Increases and decreases in excitatory postsynaptic currents (EPSCs) mediated by nicotinic and muscarinic agonists respectively have also been reported in layers II/III pyramidal neurons of rat prefrontal cortex (Vidal and Changeux 1989, 1993). Interneurons can be directly excited by nAChR activation in hippocampus (Frazier et al. 1998; Jones and Yakel 1997) and neocortex (Christophe et al. 2002; Porter et al. 1999; Xiang et al. 1998). The highest laminar densities of cholinergic axons and varicosities are found in layer I of the neocortex (Mechawar et al. 2000), which contains sparsely distributed nonpyramidal neurons (Zhou and Hablitz 1996). Such dense cholinergic innervation of layer I suggests that this layer may be a major site of cholinergic modulation of neocortical networks.

In the presence of AMPA and N-methyl-d-aspartate (NMDA), receptor antagonists to block EAA-mediated synaptic transmission, 4-aminopyridine (4-AP) produces depolariz-
ing responses in the neocortex (Aram et al. 1991; Avoli et al. 1994; Benardo 1997). Such responses are thought to arise from synchronized activity in interneurons mediated by GABA<sub>A</sub> receptors and are referred to as GABA waves. Electrophysiological (Yang and Benardo 2002) and voltage-sensitive dye imaging experiments (DeFazio and Habilitz 2005) suggest that only the superficial layers of the cortex support these responses. Because interneurons in the neocortex can be excited via activation of nAChRs (Christophe et al. 2002; Porter et al. 1999; Xiang et al. 1998), ACh may have a role in synchronizing interneuron activity in the neocortex. Using voltage-sensitive dye imaging, we show that endogenous ACh can increase synchronized interneuron activity in local inhibitory circuits in rat prefrontal cortex. This action of ACh requires activation of α4β2-like nAChRs. A preliminary account of these findings has been published in an abstract form (Bandyopadhyay et al. 2004).

**METHODS**

**Preparation of brain slices**

Brain slices from 20- to 42-day-old Sprague-Dawley rats were used. Rats were housed and handled according to the guidelines of the National Institutes of Health on Laboratory Animal Resources. Prior approval from the UAB Institutional Animal Care and Use Committee was obtained for all experimental protocols. Rats were decapitated under ketamine anesthesia (100 mg/kg ip). After quick dissection, rat brains were placed in ice-cold low-calcium saline (in mM) 125 NaCl, 3.5 KCl, 26 NaHCO<sub>3</sub>, 10 d-glucose, 3 MgCl<sub>2</sub>, and 1 CaCl<sub>2</sub>. Coronal brain slices (300 μm thick) containing the prefrontal cortex were cut using a Vibratome (Ted Pella, Redding, CA), incubated in recording saline containing (in mM) 125 NaCl, 3.5 KCl, 26 NaHCO<sub>3</sub>, 10 d-glucose, 2.5 CaCl<sub>2</sub>, and 1.3 MgCl<sub>2</sub>) for 45 min at 37°C, and then kept at room temperature until recording. The solution was bubbled with a gas mixture containing 95% O<sub>2</sub>-5% CO<sub>2</sub> to maintain pH around 7.4. Each slice was transferred before recording to a second incubation chamber where they were incubated at room temperature for 30 min in recording saline containing 4-AP (100 μM) and the EAA receptor antagonists D-2-amino-5-phosphonovaleric acid (d-APV; 20 μM) and 6-cyano-7-nitroquinoloxalin-2,3-dione (CNQX; 10 μM). Slices were subsequently transferred to a recording chamber continuously perfused (3 ml/min) with recording saline (bubbled with 95% O<sub>2</sub>-5% CO<sub>2</sub> gas mixture) containing 4-AP (100 μM), d-APV (20 μM), and CNQX (10 μM).

**Electrophysiological and optical recordings**

Neurons in slices were visualized using a Zeiss Axioskop FS (Carl Zeiss, Thornwood, NY) microscope equipped with Nomarski optics, a ×40 water-immersion lens and infrared illumination. Layer I neurons were identified by their subpial location. Biocytin labeling was used to confirm neuronal location and identity. Whole cell recordings were obtained as described previously (Bandyopadhyay et al. 2005). Patch electrodes were pulled from borosilicate glass capillary tubes and filled with intracellular solution containing (in mM) 125 K-gluconate, 10 KCl, 10 HEPES, 2 Mg-ATP, 0.2 Na-GTP, and 0.5 EGTA (pH = 7.3; osmolality = 290 mosM). When filled with this intracellular solution, the patch electrodes had resistances between 3 and 5 MΩ. Series resistance was <20 MΩ in all experiments and was not compensated. Recordings were discontinued if series resistance increased by >20%. The excitatory amino acid (EAA) receptor antagonists d-APV (20 μM) and CNQX (10 μM) were bath applied to block NMDA and AMPA receptor mediated EPSPs. Bath application of 4-AP (100 μM) in the presence of the EAA antagonists was used to unmask GABA waves in the slice. Square wave current pulses (100–400 μA in amplitude and 100 μs in duration) delivered at a frequency of 1/min via a bipolar stimulating electrode positioned in middle cortical layers were used to evoke GABA responses. All recordings were done at 32 ± 1°C. Signals were recorded using a MultiClamp 700A amplifier (Molecular Devices, Sunnyvale, CA), filtered at 5 kHz and digitized at 10–20 kHz via a Digidata 1200B interface (Molecular Devices).

The voltage-sensitive fluorescent dye [N-3-(triethylammonium)propyl]-1-4-[(p-diethylaminophenyl)butadienyl]pyridinium dibromide; RH 414; Molecular Probes, Eugene, OR) (Grinvald et al. 1988) was used for imaging experiments. Slices were incubated in recording saline containing (in μM) 30 RH 414, 100 4-AP, 20 d-APV, and 10 CNQX for 45–60 min at room temperature. Individual slices were then placed in the recording chamber on the stage of the microscope (Axiovert 135TV, Carl Zeiss) used for optical recording. The recording chamber was continuously perfused with recording saline (3 ml/min) containing 4-AP (100 μM), d-APV (20 μM), and CNQX (10 μM). Slices were allowed to sit in the recording chamber for ≈30 min prior to recording to wash out excess dye. A bipolar stimulating electrode positioned in middle cortical layers was used to evoke activity in the slices. The frequency of stimulation was 1/min. Local field potentials were recorded with an extracellular glass electrode (filled with extracellular saline) in superficial cortical layers. All imaging experiments were performed at 32 ± 1°C. A photodiode array containing 464 diodes arranged in a hexagonal fashion (Neuroplex, Red Shirt Imaging, Fairfield, CT) was used to detect changes in fluorescence due to activity in the brain slices, as described previously (Bandyopadhyay et al. 2005). The resting light intensity measured for each detector was used to normalize fluorescence measurements. Correction for dye bleaching was done using measurements taken in the absence of stimulation. All optical signals are represented as changes in fluorescence over resting fluorescence (ΔF/ΔF<sub>0</sub>) where F is the resting fluorescence and ΔF is the change in fluorescence with activity). The dye RH 414 responds to membrane depolarization with a decrease in fluorescence. Decreases in fluorescence are plotted as upward deflections in all figures. Pseudocolor images were created from the data for visualizing spatiotemporal patterns of activity in the slice. A fixed pseudocolor scale was used for all frames in a given figure. Slices were stained with cresyl violet to allow identification of the relationship of the optical signals with the cortical laminar.

**Drug application**

Neostigmine, an ACh esterase inhibitor, was used to decrease the degradation of endogenous ACh released on electrical stimulation in the slice. 1,1-dimethyl-4-phenyl-piperazinum iodide (DMPP) was used as a selective nAChR agonist, whereas dihydro-β-erythroidine (DHβE) and methyllycaconitine (MLA) were used as nicotinic antagonists. All the drugs were applied by adding to the bath solution. After recording control responses, neostigmine or DMPP were bath applied for 10–15 min. In all experiments in which antagonists were tested, the antagonists were present in both control and agonist-containing solutions unless mentioned otherwise. Drugs were stored in frozen stock solutions and dissolved in the saline prior to each experiment. 4-AP, DMPP, MLA, neostigmine, and tetrodotoxin were purchased from Sigma (St. Louis, MO) and d-APV and CNQX from Tocris Cookson (Ellisville, MO).

**Data analysis and statistics**

Data from imaging experiments were analyzed as described previously (Bandyopadhyay et al. 2005). A region of interest (ROI) in the control recording was first chosen. The ROI included photodiodes detecting visually obvious activity along with surrounding diodes detecting no apparent activity. The baseline noise level was calculated as the average of peak signal amplitudes (peak ΔF/ΔF<sub>0</sub>) of five diodes.
outside the ROI with no obvious signal. Peak signal amplitude above twice that of the baseline noise was chosen as the threshold value for activity. Diodes in the ROI showing peak signal amplitudes above the threshold were selected for analysis. The sum of the peak signal amplitudes of these diodes are referred to as the “peak activity.” The estimate of the duration of activity was obtained from the time interval between the first and the last frames of an acquisition showing activity. These parameters were measured in images taken before and 10 min after drug application in all the imaging experiments.

Student’s t-test was used for statistical comparison (OriginPro 7.0 software) before and after drug application. $P < 0.05$ was considered significant. Data are expressed as means ± SE.

RESULTS

Evoked GABA waves

Whole cell current-clamp recordings were obtained from layer I neurons ($n = 5$) in the PFC. Interneurons were identified as fast spiking cells based on action potential width and response to depolarizing current pulses. Biocytin labeling confirmed location in layer I. Intracortical stimulation was used to evoke activity in the presence of n-APV (20 μM) and CNQX (10 μM) to block NMDA and AMPA receptor-mediated excitation and 4-AP (100 μM) to enhance transmitter release in response to stimulation. Depolarizing responses with superimposed action potentials of variable amplitudes were observed at resting membrane potentials (−73 ± 2 mV, $n = 5$). Response amplitudes increased when cells were hyperpolarized by current injection (Fig. 1A, top trace). Such responses are known to be mediated by GABA$_{A}$ receptors (Aram et al. 1991; Benardo 1997). Bath application of bicuculline (10 μM) blocked the response ($n = 5$, results not shown). Whole cell responses were similar in time course to the local field potentials recorded from the superficial layers of the slice (Fig. 1A, middle trace) as well as to the optical signals obtained from neocortical slices imaged with the voltage-sensitive dye RH 414 (Fig. 1A, bottom trace). Voltage-sensitive dye imaging has the advantage of detecting activity from a large area of a brain slice suitable for studying activity in a network of neurons. Therefore imaging with the voltage-sensitive dye RH 414 was utilized to study the effect of stimulus-evoked release of endogenous ACh on spatiotemporal pattern of GABA waves in the neocortex.

Imaging of GABA waves in the neocortex

For imaging, a hexagonal photodiode array containing 464 diodes was utilized. A ×5 objective was used to cover a large area of the slice (3.5 × 3.5 mm) reaching from the pia to the white matter. Intracortical stimulation in the presence of n-APV (20 μM), CNQX (10 μM), and 4-AP (100 μM) was used to evoke GABA waves. Figure 1B shows a pseudocolor spatial map of such a response superimposed on the cresyl violet-stained image of the slice. The activity was more intense in the upper layers of the neocortex and the spread of activity was more marked in the horizontal direction as described previously (DeFazio and Hablitz 2005). The activity was abolished by tetrodotoxin (1 μM) ($n = 4$, results not shown) indicating a dependence on action-potential-dependent transmitter release.

Stimulus-evoked release of endogenous ACh enhances GABA waves in the neocortex

The spatiotemporal distribution of GABA wave activity is shown in Fig. 2A. Pseudocolor images in each panel show the spatial distribution of dye signals ($\Delta F/F$) at a given point in time. Pseudocolor scaling was the same for all frames. Warm colors represent larger-amplitude dye signals, i.e., high levels of activity. The pial surface is up in each panel. The first panel is prior to the stimulus and additional panels are shown at 50-ms intervals. The control images acquired using weak intracortical stimulation show an area of activity that appeared near the stimulating electrode and persisted for 523 ± 26 ms ($n = 13$; Fig. 2A). The largest-amplitude dye signals were observed in the superficial layers of the cortex, and the signal amplitudes gradually decreased in the deeper layers. The activity spread horizontally through the neocortex in both directions. The extent of spread was variable with 54% of the slices showing spread of activity beyond the imaged area. Similar patterns of activity were seen in experiments ($n = 10$) with more mature rats (4–6 wk old). In these older animals, 60% of the slices had widespread activity extending beyond the imaged area.

To study the effect of endogenous ACh on GABA waves, activity was evoked in the presence of the ACh esterase inhibitor neostigmine (10 μM). At this concentration, neostigmine has been shown to have ACh esterase inhibitor activity in rat brain slices from ventral tegmental area (Calabresi et al. 1997). Results not shown).
Effect of ACh on GABA waves is mediated by nAChRs

To determine the cholinergic receptor type (muscarinic or nicotinic) involved in the enhancement of GABA waves in the neocortex, we tested the effect of the muscarinic ACh receptor antagonist atropine (1 µM). The enhancement by neostigmine (10 µM) of stimulus-evoked GABA waves was not blocked by atropine (1 µM; Fig. 3). In the presence of atropine (1 µM), bath application of neostigmine led to an enhancement of activity (n = 7) as seen in experiments without atropine. Peak activity level was significantly higher (0.54 ± 0.09 vs. 0.85 ± 0.20; n = 7; P < 0.05) and duration of activity was significantly prolonged (497 ± 49 vs. 677 ± 25 ms; n = 7; P < 0.05) when neostigmine (10 µM) was applied in continued presence of atropine (1 µM; Fig. 5).

We next used the selective nAChR agonist DMPP under experimental conditions similar to those described in the preceding text for the neostigmine experiments to determine if the cholinergic enhancement of GABA waves in neocortex was mediated by nAChRs. The selective nAChR agonist DMPP (10 µM) gave rise to the same kind of enhancement of activity (n = 4 of 7 slices tested) as seen after neostigmine application. Peak activity level was significantly higher (0.28 ± 0.05 in control vs. 0.34 ± 0.05 after DMPP; n = 4; P < 0.05) and duration of activity was significantly prolonged (599 ± 95 ms in control vs. 791 ± 93 ms after DMPP; n = 4; P < 0.05) after DMPP application (Fig. 5). Atropine (1 µM) did not have any influence on the effect of DMPP (10 µM) on stimulus-evoked GABA responses (Peak activity: 0.41 ± 0.03 in atropine vs. 0.49 ± 0.05 after DMPP in presence of atropine; n = 4; P < 0.05; duration of activity: 503 ± 33 ms in atropine vs. 663 ± 21 ms after DMPP in presence of atropine; n = 4; P < 0.05). These data suggest that the effect of ACh on GABA waves was mediated by nAChRs.

Cholinergic enhancement of GABA waves require α4β2-like nAChRs

Homomeric α7-like and heteromeric α4β2-like nAChRs are the predominant nicotinic receptor subtypes in the neocortex. To determine the nAChR subtype mediating the effect of ACh
FIG. 3. The muscarinic antagonist atropine does not block the effect of neostigmine. A and B: spatiotemporal pattern of optical signals associated with GABA waves. Responses were evoked by intracortical stimulation under control conditions (A) and after bath application of 10 μM neostigmine (B) for 10 min, respectively. The muscarinic antagonist atropine (1 μM) along with t-APV (20 μM), CNQX (10 μM), and 4-AP (100 μM) were present in the bathing solution under control conditions and after neostigmine application. C: dye signals from 3 representative diodes show that an increase in the amplitude of the optical signal was observed with neostigmine (10 μM) application despite the presence of the muscarinic antagonist atropine (1 μM) in bath.

FIG. 4. The nicotinic antagonist dihydro-β-erythroidine (DHβE) blocks the cholinergic enhancement of depolarizing GABA waves in rat prefrontal cortex. A and B: spatiotemporal pattern of depolarizing GABA waves evoked by intracortical stimulation under control condition and after bath application of neostigmine (10 μM) for 10 min, respectively. The nicotinic antagonist DHβE (10 μM) along with t-APV (20 μM), CNQX (10 μM), and 4-AP (100 μM) were present in the bathing solution all through the experiment. C: dye signals from 3 representative diodes show no change in amplitudes of depolarization before and after application of neostigmine (10 μM) in the presence of DHβE (10 μM).
observed, we used the nicotinic antagonists MLA and DHβE known to selectively block α7-like and α4β2-like nAChRs, respectively. The α7 subunit-selective nAChR antagonist MLA (50 nM) was not effective in blocking the neostigmine effect (Fig. 5). Significant increases in peak activity (0.27 ± 0.01 vs. 0.34 ± 0.01; n = 5; P < 0.05) and duration of activity (420 ± 49 vs. 587 ± 17 ms; n = 5; P < 0.05) were still observed when neostigmine (10 μM) was applied in presence of MLA (50 nM). DHβE (10 μM), at this concentration known to selectively antagonize α4β2-like nAChRs (Alkondon and Albuquerque 2001; Alkondon et al. 1999), prevented the neostigmine-induced enhancement of GABA waves (Fig. 4). No significant changes in peak activity (0.37 ± 0.04 vs. 0.35 ± 0.04; n = 5; P > 0.05) or duration (326 ± 55 vs. 339 ± 61 ms; n = 5; P > 0.05) of GABA waves evoked by intracortical stimulation under this condition (Fig. 5). On the other hand, application of DHβE (10 μM) resulted in a significant decrease in both peak activity (0.52 ± 0.11 vs. 0.46 ± 0.10; n = 5; P < 0.05) and duration (443 ± 84 vs. 345 ± 73 ms; n = 5; P < 0.05) of evoked GABA waves (Fig. 5).

**Discussion**

The main findings in this study are that stimulus-evoked release of endogenous ACh can enhance the amplitude, duration, and spread of depolarizing GABA waves in rat neocortex. Our results indicate that activation of nAChRs, presumably α4β2-like nAChRs, can enhance synchronization of interneurons in the neocortex.

**Neocortical GABA waves**

Evidence for GABA_A receptor-mediated synchronous activity in interneurons was first reported by Aram et al. (1991) after application of the convulsant agent 4-AP in the presence of EAA receptor antagonists. Similar events have also been described in human neocortical slices (Avoli et al. 1994). Imaging with voltage-sensitive dyes has shown that these discharges can give rise to widespread depolarization in slices from hippocampus (Sinha and Saggau 2001) and neocortex (DeFazio and Hablitz 2005). GABA_A receptor-dependent depolarizing responses have been recorded from both pyramidal neurons and interneurons in rat neocortex (DeFazio and Hablitz 2005; Keros and Hablitz 2005). Ectopic action potentials (abruptly arising spikes of variable amplitudes occurring below normal spike threshold) during depolarizing GABA responses are more likely to occur in layer I interneurons and may play a role in synchronizing discharge from these interneurons (Benardo 1997; Keros and Hablitz 2005). Synaptic (Tamas et al. 1998; Zhou and Hablitz 1996) and electrical (Chu et al. 2003; Draguhn et al. 1999; Galaretta and Hestrin 1999) coupling of neocortical interneurons facilitates such synchronization. We show here that GABA_A receptor-dependent depolarizing responses associated with action potentials of variable amplitudes can be recorded from layer I interneurons. GABA waves in whole cell recordings correlate well with the local field potentials and the optical signals recorded from individual diodes in voltage-sensitive dye imaging experiments, suggesting that such imaging can be a powerful tool to study spatio-temporal pattern of synchronized interneuron activity in brain slices.

One possible mechanism for GABA waves in mature cortex may be elevation of extracellular potassium ions leading to intracellular chloride accumulation due to influx of chloride via potassium-coupled chloride transporters (DeFazio and Hablitz 2005; DeFazio et al. 2000). Elevations of extracellular potassium during GABA waves (Louvel et al. 2001) and enhancement of GABAergic responses by 4-AP occur preferentially in upper cortical layers (Barkai et al. 1995; Yang and Benard 2002). Elevations in extracellular potassium could enhance ACh release from cholinergic terminals.

**Cholinergic modulation of synaptic transmission in neocortex**

Although nAChRs are known to mediate fast excitatory synaptic transmission in the hippocampus (Alkondon et al. 1998; Frazier et al. 1998; Jones and Yakel 1997) and the neocortex (Chu et al. 2000; Roerig et al. 1997), the major role of ACh in the brain appears to be modulatory. Both α4β2-like and α7-like nAChRs have been shown to modulate glutamate mediated synaptic transmission (Gioanni et al. 1999; Gray et al. 1996; McGehee et al. 1995; Radcliffe and Dani 1998) and GABA (Alkondon et al. 1997, 1999). Glutamate release from thalamocortical terminals onto pyramidal cells in rat prefrontal cortex evoked by 4-AP is increased by 2-AP or DHβE (10 μM) but not MLA (50 nM), and the effects are blocked by atropine (1 μM). MLA (50 nM), DHβE (10 μM), or atropine (1 μM) each blocks depolarizing responses associated with action potentials of variable amplitudes occurring below normal spike threshold during depolarizing GABA responses.

**Fig. 5.** Summary of effects of cholinergic drugs on evoked GABA waves in rat prefrontal cortex. A: percentage change in the peak activity (see METHODS). B: percentage change in the duration of activity. Error bars indicate SE. Neo, neostigmine (10 μM), n = 13; Neo + Atro, neostigmine (10 μM) in the presence of atropine (1 μM), n = 7; DMPP, 1,1-dimethyl-4-phenyl-piperazinium iodide (DMPP, 10 μM), n = 4; DMPP + Atro, DMPP (10 μM) in the presence of atropine (1 μM), n = 4; Neo + DHBE, neostigmine (10 μM) in the presence of DHβE (10 μM), n = 5; Neo + MLA, neostigmine (10 μM) in the presence of methyllycaconitine (MLA, 50 nM); DHBE, DHβE (10 μM), n = 5; MLA, MLA (50 nM), n = 5. *, significance at P < 0.05.
cortex has been shown to be increased by presynaptic nAChR activation (Gioanni et al. 1999; Lambe et al. 2003; Vidal and Changeux 1989, 1993); α4β2-like nAChRs have been implicated based on pharmacological sensitivity and the fact that the effect is absent in transgenic mice lacking β2 subunits (Lambe et al. 2003). Interneurons in rat prefrontal cortex can be directly excited by stimulation of postsynaptic α4β2-like and α7-like nAChRs (Christophe et al. 2002; Porter et al. 1999). Such excitation is confined to a subset of interneurons in deeper (II-V) layers (Porter et al. 1999), whereas all types of interneurons in layer I are excited (Christophe et al. 2002). Human neocortical interneurons also have functional nAChRs and stimulation of presynaptic α4β2-like nAChRs results in synchronized GABA release from several GABAergic synapses (Alkondon et al. 2000). Synchronization of GABA release from interneurons in upper layers of prefrontal cortex may underlie the facilitatory effect of neostigmine on GABA waves observed in the present imaging experiments. Because neostigmine blocks degradation of ACh, its effect is usually attributed to a prolonged action of ACh. Although ACh esterase inhibitors can act as allosteric modulators of nAChRs (Maelicke et al. 1993; Pereira et al. 1994; Schrattenholz et al. 1996; Storch et al. 1995), such modulation is insensitive to blockade by competitive nAChR antagonists like DHβE (Pereira et al. 1994; Storch et al. 1995). A direct effect of neostigmine on nAChRs can be excluded because DHβE (10 μM) blocked neostigmine-induced enhancement of evoked GABA waves. DHβE (10 μM) also depressed GABA waves evoked in the absence of neostigmine. Direct inhibitory effects of neostigmine on nAChRs, if present, would have attenuated the ACh-induced enhancement of GABA waves. These observations lend support to the fact that endogenously released ACh is involved in the enhancement of evoked GABA waves seen in this study.

The cholinergic enhancement of GABA waves was mediated by nAChRs because the effect was mimicked by the nAChR agonist DMPP (10 μM). The DMPP-induced enhancement could be blocked by the nicotinic antagonist DHβE (10 μM) but not by the muscarinic antagonist atropine (1 μM). The α4β2 subtype of nAChRs was involved because the nAChR antagonist DHβE (10 μM) predominantly blocks α4β2-like nAChRs (Alkondon and Albuquerque 2001; Alkondon et al. 1999), and the nicotinic effect was insensitive to blockade by the antagonist MLA (50 nM) selective for α7-like nAChRs (Alkondon et al. 1992).

ACh, synchronized interneuron activity, and network oscillations

Networks of GABAergic interneurons can generate synchronous oscillations (Blatow et al. 2003; Buzsáki and Chrobak 1995; Fisahn et al. 1998). Synchronization of a mutually interconnected network of interneurons can occur via recurrent collaterals as a result of depolarizations mediated by synaptically activated GABA<sub>A</sub> receptors as well as electrical coupling between the interneurons (Beierlein et al. 2000; Benardo 1997; Galaretta and Hestrin 1999; Michelson and Wong 1994; Skinner et al. 1999). Oscillations arising out of synchronized interneuron activity may provide a temporal structure for correlated firing of a group of neurons that encodes information in a cortical circuit and phase their firing activity. GABAergic interneurons in hippocampus have the ability to phase spontaneous firing and subthreshold oscillations in pyramidal cells at theta frequencies (Cobb et al. 1995). Association of hippocampal theta rhythm with exploratory behavior in rodents suggests its involvement in spatial learning and memory (Bland 1986; Huerta and Lisman 1993; Larson et al. 1986). Theta oscillations have also been reported in the neocortex (Bao and Wu 2003; Blatow et al. 2003; Raghavachari et al. 2001; Sarntinirat et al. 1998), and bursting discharges from cholinergic basal forebrain neurons have been shown to be associated with theta oscillations (Lee et al. 2005). Cholinergic induction of theta oscillations has been reported in a chemically and electrically coupled network of interneurons in mouse neocortex (Blatow et al. 2003). Thus cholinergic enhancement of synchronized interneuron activity observed in this study may have a role in theta frequency oscillations in rat neocortex.

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