Neuronal nicotinic acetylcholine receptors (nAChRs) expressed by neurons of the neocortex are known to play a role in higher brain functions. Electrophysiological studies of neocortical neurons provided evidence that functional nAChRs are present on the axonal presynaptic terminals, on the somata and on dendrites of gamma-aminobutyric acid (GABA)ergic inhibitory interneurons. However, it is not clear if pyramidal neurons express functional postsynaptic nAChRs. Therefore, we investigated the action of locally applied acetylcholine (ACh) on layer 5 pyramidal neurons in the rat neocortex in vitro. In the presence of atropine, tetrodotoxin, glutamate receptor antagonists, and GABA_A receptor antagonists, ACh induced membrane depolarizations which were generated by membrane inward currents consisting of a fast and a slow component. Analysis of the electrophysiological properties, the pharmacological characteristics, and the desensitization behavior of the 2 current components revealed that they were mediated by at least 2 different subtypes of the nAChR, most likely the α7-like and the α4β2-like subtype. The expression of nAChRs in neocortical pyramidal cells raises the possibility that these neurons generate nicotinic excitatory postsynaptic potentials, thereby influencing cell excitability. Furthermore, because most nAChRs are permeable to calcium, they may modulate synaptic transmission and neuronal plasticity via a calcium-dependent postsynaptic mechanism.

Keywords: layer 5, neocortex, nicotinic acetylcholine receptors, postsynaptic membrane currents, pyramidal cells

Introduction
The cholinergic innervation of the neocortex originates from the acetylcholine (ACh)-synthesizing neurons of the basal forebrain (Mesulam et al. 1983; Eckenstein et al. 1988; Lysakowski et al. 1989; Descarries et al. 2005; Henny and Jones 2008). Activation of the cholinergic basal forebrain neurons in vivo leads to alterations of neocortical activity, including a desynchronization of the electroencephalogram and facilitation of sensory inputs to the neocortex (Semba 2000; Metherate 2004). ACh released upon stimulation of the basal forebrain modulates the activity of neocortical networks via muscarinic and nicotinic ACh receptors (Semba 2000; Sarter et al. 2003; Metherate 2004). Behavioral and clinical studies revealed that activation of cholinergic basal forebrain afferents influences higher central nervous system (CNS) functions including attention, sleep, learning, memory, pain perception, as well as cognition, and that nicotinic ACh receptors (nAChRs) expressed by neocortical neurons are involved in these regulatory functions (Picciotto et al. 2000; Sarter et al. 2003; Gotti and Clementi 2004; Newhouse et al. 2004b; Levin et al. 2006). In addition, many lines of evidence suggest that neocortical nAChRs contribute to the pathogenesis of brain disorders such as epilepsy, Alzheimer’s disease, and schizophrenia (Sutor and Zolles 2001; Raggenbass and Bertrand 2002; Gotti and Clementi 2004; Newhouse et al. 2004a; Levin et al. 2006).

Nicotinic AChRs seem to regulate the transmitter release from nerve terminals via preterminal or presynaptic receptors (Wonnacott 1997; Jones et al. 1999). In addition, neocortical inhibitory interneurons are endowed with functional postsynaptic nAChRs (McCormick and Prince 1986; Nicoll et al. 1996; Roerig et al. 1997; Xiang et al. 1998; Porter et al. 1999; Alkondon et al. 2000; Chu et al. 2000; Christophe et al. 2002; Couey et al. 2007). Activation of these receptors induces excitation of inhibitory interneurons and a consequent inhibition of pyramidal cells innervated by these interneurons.

There is still controversy concerning the possible expression of functional postsynaptic nAChRs by neocortical pyramidal cells. Roerig et al. (1997) detected nicotinic excitatory postsynaptic currents (EPSCs) in both interneurons and pyramidal cells of the ferret neocortex. Furthermore, nicotinic excitatory postsynaptic potentials (EPSPs) and EPSCs have been observed in pyramidal neurons of the rat neocortex (Chu et al. 2000). Other groups, however, were not able to detect nAChR-mediated postsynaptic actions in pyramidal neurons of the rodent neocortex (McCormick and Prince 1986; Vidal and Changeux 1993; Nicoll et al. 1996; Gil et al. 1997; Aramakis and Metherate 1998; Xiang et al. 1998; Porter et al. 1999; Couey et al. 2007). On the other hand, several immunohistochemical studies demonstrated the expression of α7-subunits at the postsynaptic site of asymmetric synapses of neocortical as well as hippocampal pyramidal cells (Lubin et al. 1999; Fabian-Fine et al. 2001; Levy and Aoki 2002), suggesting the existence of postsynaptic nAChRs in these neurons. In the present study, we investigated the actions of locally applied ACh on layer 5 pyramidal neurons...
of the rat neocortex in vitro. We found that these neurons express at least 2 subtypes of functional postsynaptic nAChRs and that the activation of these receptors results in inward currents leading to membrane depolarizations.

Material and Methods

Preparation of Brain Slices

The preparation of brain slices was performed in accordance with the EC Council directives for animal care. Neocortical slices were prepared from the brains of juvenile Wistar rats (15–25 days old) of either sex. The animals were deeply anaesthetized with isoflurane (Forene, Abbott, Wiesbaden, Germany) or enflurane (Enfluran-Baxter, Baxter Deutschland GmbH, Unterschleißheim, Germany). Following the decapitation of an animal, the brain was removed from the skull and stored for one minute in ice-cold artificial cerebrospinal fluid (ACSF). Then, the hemispheres were separated and fixed onto the stage of a vibratome (VT 1000S, Leica Instruments, Nussloch, Germany). Coronal slices (thickness 300 μm) were cut from both the frontal and the somatosensory cortex, and stored in ACSF at 35 °C for 30 min. After an additional 90 min of incubation at room temperature, one slice was transferred to the recording chamber, stabilized by means of a nylon mesh and superfused with ACSF at a flow rate of 1.5–2 ml/min. The ACSF consisted of (in mM): 125 NaCl, 3 KCl, 1.25 NaH2PO4, 2 CaCl2, 2 MgCl2, 25 NaHCO3, and 25 glucose. The solution was saturated with 95% O2 and 5% CO2, resulting in a pH of 7.4. Most recordings were performed at room temperature (∼20 °C). In 13 experiments, the recording temperature was raised to 28 °C. At this temperature, the effects of ACh on pyramidal cells were similar to those obtained at room temperature. Therefore, the data acquired at different experimental conditions were pooled. The recording chamber was mounted onto the stage of an upright microscope (Axioskop FS, Zeiss, Oberkochen, Germany). In order to visualize single neurons, the microscope was equipped with differential interference contrast optical devices (DIC), an infrared filter and an infrared-sensitive CCD camera (C2-400, Hamamatsu Photonics K.K., Hamamatsu-City, Japan) connected to a video monitor. In addition, epifluorescence devices (Zeiss filter set 38 HE, 495 nm/525 nm) were incorporated into the microscope in order to visualize neurons filled with fluorescent dyes.

Electrophysiological Techniques

The whole cell mode of the patch-clamp technique was used to investigate ACh-induced membrane potential changes and membrane currents. Patch-clamp pipettes were produced from borosilicate glass capillaries (o.d. 1.5 mm, i.d. 1.17 mm, Clark Electromedical Instruments, Reading, UK) and filled with a solution containing (in mM): 135 NaCl, 3 KCl, 1.25 NaH2PO4, 2 CaCl2, 2 MgCl2, 25 NaHCO3, and 25 d-glucose. The solution was saturated with 95% O2 and 5% CO2, resulting in a pH of 7.4. Most recordings were performed at room temperature (∼20 °C). In 13 experiments, the recording temperature was raised to 28 °C. At this temperature, the effects of ACh on pyramidal cells were similar to those obtained at room temperature. Therefore, the data acquired at different experimental conditions were pooled. The recording chamber was mounted onto the stage of an upright microscope (Axioskop FS, Zeiss, Oberkochen, Germany). In order to visualize single neurons, the microscope was equipped with differential interference contrast optical devices (DIC), an infrared filter and an infrared-sensitive CCD camera (C2-400, Hamamatsu Photonics K.K., Hamamatsu-City, Japan) connected to a video monitor. In addition, epifluorescence devices (Zeiss filter set 38 HE, 495 nm/525 nm) were incorporated into the microscope in order to visualize neurons filled with fluorescent dyes.

Membrane potential changes and membrane currents mediated by nAChRs were evoked by pressure application of ACh from glass pipettes fabricated from borosilicate glass capillaries (o.d. 1.5 mm, i.d. 1.17 mm, Clark Electromedical Instruments, Reading, UK, tip diameter 1–2 μm). The pipettes were filled with 1 mM ACh (acetylcholine chloride obtained from Sigma, Deisenhofen, Germany) freshly dissolved in 95% O2/5% CO2-saturated ACSF. The pH of this solution was 7.35 at room temperature. In the absence of continuous perfusion with 95% O2/5% CO2, the pH of the ACh solution changed from 7.35 to 7.42 within one hour. Nevertheless, ACh-filled pipettes were replaced regularly at intervals of 1–2 h or after the end of recording from a neuron. Under visual control, the pipette tips were positioned at a distance of 10–20 μm to the somata of the neurons. ACh was applied by pressure pulses with a constant pulse strength of 0.7 bar and durations ranging between 10 and 500 ms. Applications of ACh with short pulses (10–100 ms) were performed at an interpulse interval of 30 s, and of those with long pulses (200–500 ms) at an interval of 60 s.

From all other drugs used in this study, we prepared stock solutions, either in water or, in the case of CNQX, in dimethyl sulfoxide (DMSO). Appropriate aliquots of these stock solutions were added to the bathing solution. The drug concentrations given in the text refer to the end concentration in the bathing solution. The DMSO concentration applied (0.01%) did not affect neuronal properties (see: Hablitz and Sutor 1990). Atropine (atropine sulfate), bicuculline (bicuculline methiodide), CNQX (6-cyano-7-nitroquinoxaline-2,3-dione), DHPe (dihydro-beta-erythroidine), MLA (methyllycaconitine), and TTX (tetrodotoxin) were obtained from Sigma (Deisenhofen, Germany). 2-Amino-5-pentanoic acid (D-APS) was purchased from Tocris Cookson, Ltd (Bristol, UK).

Data Analysis and Statistics

Data analysis and statistical evaluations were performed using the software packages Igor Pro (WaveMetrics, Lake Oswego, Oregon) and Prism 3 (Graphpad Software, San Diego, CA). Absolute values are given as mean ± SD, normalized values as mean ± SEM. Tests of statistical significances were performed by applying the paired or unpaired 2-tailed Student’s t-test, the one-sample t-test, the nonparametric Mann-Whitney test, the One-way ANOVA (including Bonferroni’s multiple comparison test), the ANOVA for repeated measures (including Bonferroni’s Multiple Comparison test) or the Kruskal-Wallis test (including Dunn’s multiple comparison test).

Results

Identification and Properties of Layer 5 Pyramidal Neurons

The effects of local ACh application were investigated in pyramidal neurons located in layer 5 of the rat frontal and somatosensory cortex. These neurons were identified by their position in the slice preparation and by assessment of their morphology using infrared-DIC-microscopy. The neurons were characterized by their large pyramidal-shaped somata and by their clearly discernible apical dendrites (Fig. 1A, see Supplementary Fig. S1A). After labeling with the fluorescent dye Alexa 488 (n = 5), the apical dendrites could be visualized up to the pial surface of the slice (see Supplementary Fig. S1C,D). Pyramidal neurons responded to the intracellular injection of suprathreshold depolarizing current pulses with slowly adapting trains of action potentials (Fig. 1B, see Supplementary Fig. S1B) and were therefore classified as regular spiking pyramidal neurons (McCormick et al. 1985; Kawaguchi 1993). The mean resting membrane potential of these cells was −63.6 mV (±4.5 mV, n = 105) and the mean input resistance was 11.28 MΩ (±4.69 MΩ, n = 102).
Isolation of nAChR-Mediated Postsynaptic Responses in Layer 5 Pyramidal Cells

In the absence of any cholinergic receptor antagonist, the application of ACh with short pulses (50 ms) to the somata of pyramidal neurons resulted in long-lasting membrane potential changes (Fig. 1C) consisting of 3 components: 1) an initial depolarization, which sometimes elicited single action potentials, 2) a variable period (500 ms–4 s) of sustained depolarization characterized by a lack of action potentials, and 3) a late depolarizing component which evoked a discharge of action potentials.

In order to block muscarinic AChRs, we added atropine (1 μM) to the ACSF. In the presence of this muscarinic antagonist, the application of ACh with short pulses (e.g., 25 ms, Fig. 2A) induced a relatively fast rising depolarization of 2–20 mV in amplitude and a duration ranging between 50 and 150 ms. This initial depolarization was followed by a slowly decaying depolarization of maximum 5 mV in amplitude and a duration of up to 2 s (Fig. 2A). An increase in the pressure pulse duration (e.g., from 25 to 50 ms, Fig. 2B) led to a small increase in the amplitude of the initial depolarization and to a slight prolongation of the slow decay. Sometimes, the initial ACh-induced depolarizations were sufficient to evoke action potentials (Fig. 2B). In about 90% of the neurons, the application of ACh in the presence of atropine gave rise to the discharge of small synaptic potentials during the slowly decaying component of the response (Fig. 2C). These synaptic events could be blocked by the application of a cocktail consisting of the glutamate receptor antagonists CNQX and D-AP5, the GABA<sub>A</sub> receptor antagonist bicuculline and the sodium channel blocker TTX (Fig. 2D). The residual ACh-induced responses comprised a fast rising and fast decaying initial component (amplitude: 2–8 mV, duration: 100–200 ms) and a slowly decaying second component, which could last up to 10 s (Fig. 2D,E, left panel). Because the antagonists and blockers applied caused an almost complete "functional synaptic isolation" of the neurons, it is reasonable to conclude that the residual ACh-induced responses were mediated by postsynaptic nAChRs expressed in layer 5 pyramidal cells. As we concentrated our investigations on postsynaptic nicotinic responses of pyramidal cells to ACh, all following experiments were carried out in the presence of atropine (1 μM), CNQX (10 μM), D-AP5 (10–20 μM), bicuculline (10 μM), and TTX (0.6–1 μM). The concentration of atropine applied (1 μM) was sufficient to block all muscarinic receptors in the slice preparation (see also Porter et al. 1999; Christophe et al. 2002). Larger concentrations of atropine (10 μM) neither reduced the ACh-induced currents nor uncovered additional ACh effects (data not shown).

Voltage-clamp recordings in the presence of this cocktail of antagonists revealed that the ACh-induced membrane potential changes were produced by membrane inward currents consisting of an initial fast component followed by a slowly decaying current (Fig. 2F, right panel). When ACh was applied with pulses of 50 ms in duration, these currents displayed an initial amplitude with a large interneuronal variability and a fast rise time (Table 1). The current decay could be fitted with a bi-exponential function yielding a fast and a slow decay time constant (Table 1). The determination of the currents' kinetic properties was performed on averages of 5 consecutively recorded signals (interval: 30 s).

Characterization of nAChR-Mediated Postsynaptic Currents in Layer 5 Pyramidal Cells

To verify that these currents were mediated by nAChRs, we investigated the effects of the subtype selective nAChR antagonists MLA (3–50 nM, n = 54) and DHβE (3–10 μM, n = 33) on the ACh-induced currents (Raggenbass and Bertrand 2002; Hogg et al. 2003). When we applied the antagonists simultaneously (Fig. 3A, 10 nM MLA and 1 μM DHβE), we observed a complete block of the membrane currents induced by short ACh pulses (50 ms) in all neurons tested (n = 5). The effects of the antagonists were at least partially reversible following washout of the compounds. In 2 neurons, we could record the time course of recovery from the antagonists' action for 90–110 min. Within this period, the recovery of the initial
amplitude was found to be almost complete (Fig. 3B), whereas the area of the current response returned to about 75% of control (Fig. 3C). In another group of cells, we applied either MLA (n = 5) or DHβE (n = 4) and tested the reversibility of the antagonists’ effects on membrane currents induced by short ACh pulses (50 ms, Fig. 3D,E). MLA (10 nM) depressed the initial amplitude of the currents by 89% (Fig. 3D, filled circles) and the area of the response by 44% (Fig. 3E, filled circles). Following a period of antagonist washout lasting for 30–40 min, the amplitude returned to 66% of control and the area to 83% of control. These effects were statistically significant (ANOVA for repeated measures, P < 0.035), the recovery from the antagonist’s action (to 83% of control, 30–40 min washout) failed to be significant (Bonferroni’s post test, P > 0.05). By contrast, the effect of DHβE on the response area was much stronger and clearly reversible. DHβE reduced the area of the current response by 80% (Fig. 3E, open circles), and following washout of this antagonist for 30–40 min, the area recovered to 47% of control. These effects of DHβE were significant (ANOVA for repeated measures, P < 0.001).

Because the ACh-induced currents were blocked by selective nicotinic receptor antagonists, these experiments provide evidence that the atropine-resistant membrane inward currents induced by application of ACh in functionally isolated layer 5 pyramidal neurons resulted from the activation of postsynaptic nACh receptors. The effects of the activation of postsynaptic nAChRs were studied in 175 pyramidal cells. Out
of this population, 122 neurons responded to the somatic application of ACh with a membrane inward current consisting of a fast and a slow component. The remaining neurons did not show any response to the application of ACh. In 16 experiments, we applied ACh-free ACSF to the neurons and did not observe any membrane potential changes or membrane currents (see Supplementary Fig. S2). Therefore, we conclude that these currents were not due to mechanical artifacts produced by pressure application.

The pharmacological data shown in Figure 3D and E suggested that MLA acted predominantly on the initial part of the ACh-induced membrane currents, whereas DHβE influenced mainly later components of these responses. To further investigate this observation, we evoked nicotinic membrane currents in pyramidal neurons by ACh pressure pulses of 50 ms in duration and applied the antagonists in different sequences (n = 17). Figures 4A and B show examples of such experiments. When MLA (10 nM) was applied first, the initial fast component of the currents disappeared almost completely (Fig. 4A). The residual currents (Fig. 4A, middle panel, black trace) displayed a slow time course and could be blocked by addition of DHβE (1 μM) to the bathing solution (Fig. 4A, middle panel, gray trace). Therefore, we designated the MLA-insensitive slow current component as the DHβE-sensitive component of the total current response (Fig. 4A, right panel, black trace). The fast rising and fast decaying MLA-sensitive component (Fig. 4A, right panel, gray trace) was obtained by digitally subtracting the DHβE-sensitive component from the total membrane current (Fig. 4A, Control). A change in the application sequence (i.e., DHβE first) yielded similar results (Fig. 4B). The residual current observed in the presence of DHβE (1 μM) consisted of a fast rising and fast decaying component (Fig. 4B, middle and right panels, black traces), which was found to be abolished after addition of MLA (10 nM) to the ACSF (Fig. 4B, middle panel, gray trace). Subtraction of this MLA-sensitive component from the total current response resulted in a current with...
The possibility to pharmacologically separate the MLA-sensitive component from the DHβE-sensitive component enabled us to determine the specific kinetic properties of these 2 components (Table 1). The statistical analysis revealed that the initial current amplitude was dominated by MLA-sensitive nAChRs. DHβE-sensitive receptors contributed about 21% to this amplitude. Similarly, the fast rise time of the control response was determined by MLA-sensitive nAChRs. The rise time of the DHβE-sensitive component was found to be considerably longer than that of both the control response and the MLA-sensitive component. Although the areas of the current responses were quite variable, we determined a significantly smaller area of the MLA-sensitive component as compared with the control responses. On the other hand, the area of the DHβE-sensitive component was not significantly different from control, but also not different from that of the MLA-sensitive component. The MLA-sensitive component displayed 2 decay time constants with a fast time constant, which was significantly different from that of the control response, and a slow time constant, which differed significantly compared with control responses. By contrast, the DHβE-sensitive component decayed with only one slow time constant which was significantly longer than those of both the control response and the MLA-sensitive component.

The data described so far suggested that the nicotinic membrane currents induced in neocortical pyramidal neurons by application of ACh with pressure pulses of 50 ms in duration were composed of 2 pharmacologically distinct components, which contributed equally, but at different times to the total response. The initial part was predominantly mediated by MLA-sensitive nAChRs (Fig. 5A, left panel), the later parts by DHβE-sensitive nAChRs (Fig. 5B, left panel). When ACh was applied with pressure pulses of 500 ms in duration (Fig. 5A and B, middle panels, black traces), we observed a significant increase in a slow time course (Fig. 4B, right panel, gray trace), which represented the DHβE-sensitive component.

The investigation of the concentration–response characteristics of the 2 antagonists supported the finding that MLA predominantly suppresses a fast current component, whereas DHβE acts on a slower part of the current. Figure 4C shows the concentration–response curve for MLA. We tested different concentrations of the antagonist and found that MLA exerted a stronger action on the initial amplitude of the ACh-induced currents (Fig. 4C, closed circles) compared with its effect on the area of the currents (Fig. 4C, open circles). The difference in the inhibitory efficacy of MLA (10 nM) on amplitude and area was statistically significant ($P < 0.0004$, Mann–Whitney test). DHβE had only a weak effect on the initial amplitude of the nicotinic currents (Fig. 4D, closed circles) compared with control responses. By contrast, the inhibitory action of DHβE on the response area was much stronger (Fig. 4D, open circles). The statistical comparison between the DHβE effects on amplitude and area revealed a significance of $P < 0.0001$ (Mann–Whitney test).

Figure 4. Components of the ACh-induced nicotinic membrane currents in layer 5 pyramidal neurons. (A) Nicotinic inward currents evoked in a voltage-clamped pyramidal neuron (holding potential: −70 mV) by short ACh pressure pulses (50 ms, the time of application is indicated by the arrows above the traces). The left panel shows the Control recording. The middle panel depicts the recordings obtained in the presence of MLA (10 nM, black trace) and following additional application of DHβE (1 μM, gray trace). The right panel displays the MLA-insensitive, but DHβE-sensitive component (black trace) and the MLA-sensitive component (gray trace) superimposed. The MLA-sensitive component was obtained by subtracting the DHβE-sensitive component from the Control recording. (B) Similar experiment and analysis as described in A obtained from a different neuron. In this cell, DHβE was applied first. All traces in (A) and (B) represent the average of 5 consecutive single recordings. (C) Concentration–response curves for MLA. The relative reduction in amplitude and area with respect to control was determined and plotted as a function of the MLA-concentration applied. The data points represent mean ± SEM (2) Similar concentration–response curves for DHβE. All recordings were performed in the presence of atropine (1 μM), CNQX (10 μM), D-AP5 (10 μM), bicuculline (10 μM), and TTX (1 μM).
in the response area by a factor of 4 ($P < 0.001$, Fig. 5C). Both the initial amplitude (Fig. 5C) and the rise time of the currents remained unchanged. These findings suggested that a prolongation of the application pulse led to an enhancement mainly of the DHβE-sensitive component. To test this hypothesis, we compared the properties of nicotinic currents induced by short ACh pulses (50 ms) to that of currents elicited by long pulses (500 ms) in the same neuron. In one series, we added MLA (10 nM, $n = 5$) in order to determine the MLA-sensitive component (Fig. 5A). In the other series, we applied DHβE (1 μM, $n = 5$) for determination of the DHβE-sensitive component (Fig. 5B). These components were obtained by the subtraction procedure as described above. Figure 5A (right panel) shows the MLA-sensitive components induced by ACh pulses of 50 ms (gray trace) and 500 ms (black trace) in duration plotted on the same time scale. The statistical analysis revealed that the mean value of the area of the MLA-sensitive component increased significantly from 120.3 ± 72.6 nA ms to 248.2 ± 93.3 nA ms ($n = 5$, $P < 0.05$, Student’s $t$-test). However, the relative contribution of this area to the total response remained constant (50-ms pulse: 72.4 ± 15.0%, 500-ms pulse: 75.6 ± 4.2%) indicating that prolonged ACh application recruited predominantly DHβE-sensitive nAChRs.

**Desensitization of nAChR-Mediated Membrane Currents in Layer 5 Pyramidal Cells**

Nicotinic AChRs display a rapid and pronounced desensitization following prolonged or repeated application of nicotinic agonists (Zoli et al. 1998; Quick and Lester 2002; Guo and Lester 2007). Here, we examined the desensitization and the recovery from desensitization of the postsynaptic nicotinic currents evoked in pyramidal neurons by application of ACh. In a first set of experiments, several series of 5 consecutive short pulses (50 ms) were applied to the neuron at different interpulse intervals (Fig. 6). These series were separated by 4 min. The peak amplitudes and the areas of the responses were determined and normalized with respect to the first response in a series. The initial peak amplitude of the nicotinic membrane current decreased significantly with decreasing interpulse interval (Fig. 7A). At an interval of 5 s (Fig. 7A, open circles, $n = 8$), the first application evoked a complete response consisting of an initial fast component and a slow component (Fig. 6A). The following 4 ACh applications, however, induced only slow currents with very small amplitudes. At an

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**Figure 5.** Dependence of the ACh-induced nicotinic membrane currents on the duration of the ACh application. (A) Nicotinic currents evoked in a pyramidal neuron by application of ACh for 50 ms (left panel, black trace) and 500 ms (middle panel, black trace). Voltage-clamp recordings at a holding potential of −70 mV. The superimposed gray traces in both panels show the recordings after addition of MLA (10 nM) to the bathing solution. The right panel displays the corresponding MLA-sensitive components superimposed (black: 500 ms ACh pulse, gray: 50 ms pulse). These components were obtained by digital subtraction (see Fig. 4). The time of ACh application is indicated by the arrows above the traces. (B) Example of an experiment analogous to that described in A. In this experiment, DHβE (1 μM) was applied in order to determine the DHβE-sensitive components (right panel). Note the different time scale in the left panels of (A) and (B). All traces represent the average of 5 consecutive recordings performed at intervals of 30 s (50-ms ACh pulse) or 60 s (500-ms ACh pulse). (C) The bar diagram depicts the dependence of amplitude and area on the duration of the ACh application. The measured values were normalized with respect to the values obtained with a pressure pulse duration of 50 ms. The bars represent the mean ± SEM. All recordings were performed in the presence of atropine (1 μM), CNQX (10 μM), D-AP5 (10 μM), and TTX (1 μM).
application interval of 20 s (Figs 6C and 7A), the amplitude of the initial fast component was found to be reduced by 14% (second application) and by 13% (fifth application) in comparison to the first application of the series. These reductions in amplitude were statistically significant ($P < 0.02$, $n = 8$, one-sample $t$-test), indicating a noticeable receptor desensitization even at an application interval of 20 s. In 3 experiments, we investigated the recovery from desensitization at an application interval of 30 s and found that each ACh application in a series of 5 was able to evoke a complete response, without any detectable desensitization. These results suggest that the rapid desensitization of the nAChRs responsible for the initial fast current component recovered within a period of 20–30 s. In order to measure the desensitization of the slow current component, we determined the areas of the currents, normalized them with respect to the

Figure 6. Desensitization of nicotinic membrane currents following repetitive application of ACh. Representative current recordings following ACh application with a 50 ms pressure pulse to a layer 5 pyramidal neuron at intervals of 5 s (A), 10 s (B), 20 s (C). The recordings were performed at a holding potential of $-70 \text{ mV}$. The first, second and fifth application in a series of 5 is shown. The series were separated by 4 min. All recordings were performed in the presence of atropine (1 $\mu$M), CNQX (10 $\mu$M), D-AP5 (10 $\mu$M), bicuculline (10 $\mu$M), and TTX (1 $\mu$M).

Figure 7. Desensitization of nicotinic membrane currents following repetitive application of ACh at different intervals (A) Dependence of the initial peak current amplitude evoked by short ACh application pulses (50 ms) on the interpulse interval. Interval durations are indicated as: open circles—5 s; closed circles—10 s; open squares—15 s; closed squares—20 s. (B) Dependence of the area of the current evoked by short ACh application pulses (50 ms) on the interpulse interval. The same symbols were used in (A) and (B). (C) Nicotinic membrane currents evoked by long ACh application pulses (500 ms, holding potential: $-70 \text{ mV}$). The diagram shows the dependence of the initial peak amplitude on the interpulse interval. Durations between applications are indicated as closed squares—20 s; closed circle—30 s; open squares—60 s. (D) Dependence of the area of the currents evoked by long ACh application pulses (500 ms) on the interpulse interval. The same symbols were used in (C) and (D). All recordings were performed in the presence of atropine (1 $\mu$M), CNQX (10 $\mu$M), D-AP5 (10 $\mu$M), bicuculline (10 $\mu$M), and TTX (1 $\mu$M).
area of the first response in a series and plotted these values as a function of the number of application (Fig. 7B). At an application interval of 5 s (Fig. 7B, open circles), we found a decrease in the area by 50% (second application) and 57% (fifth application) compared with the response to the first ACh pulse. This reduction was statistically significant ($P < 0.001$, $n = 8$, one-sample t-test). At an interval of 15 s, each application in a series produced a full response, and no desensitization of the slow current component could be detected. Thus, the recovery from desensitization of the nAChRs responsible for the slow current component following short ACh pulses occurred within a time period of 10–15 s, and is faster than recovery of the fast component.

Because the slow component of the nicotinic currents was more prominent following application of ACh with long pulses, we performed a second set of experiments in which ACh was applied with pulses of 500 ms in duration. A series of 5 consecutive long pulses was delivered to the neurons at 3 different interpulse intervals (20, 30, and 60 s). The series were separated by 4 min. The peak amplitudes and the areas of the responses were determined and normalized with respect to the first response in a series. At an application interval of 20 s, the peak amplitude of the slow component was found to be reduced by 52% (Fig. 7C, closed squares, fifth application, $n = 5$). This reduction was statistically significant ($P = 0.01$, one-sample t-test). At an interval of 30 s and of 60 s, respectively, the peak amplitude of the nicotinic current did not change significantly during a series of 5 pulses ($n = 5$, Fig. 7C). The area of the current declined by maximum 38% during a series of 5 long ACh pulses applied at an interval of 20 s (Fig. 7D, closed squares). This decrease in the area was statistically significant ($P = 0.02$, $n = 5$, one-sample t-test). With application intervals of 30 and 60 s, we observed no significant changes in the current area (Fig. 7D, $n = 5$).

These experiments suggest that the nAChRs responsible for the initial fast current component and for the slow component differ in the magnitude of desensitization. To substantiate this hypothesis, we investigated the recovery from desensitization as described above in the presence of DHβE (1 μM) or MLA (10 nM) (see Supplementary Fig. S3). When ACh was applied with short pulses (50 ms) at an interval of 5 s, the MLA-sensitive current component was reduced by 92.7 ± 2.9% (peak amplitude) and 84.2 ± 2.7% (area). These reductions were significant (peak amplitude: $P = 0.003$; area: $P = 0.017$, $n = 3$, unpaired student t-test). At an application interval of 20 s, we did not observe any significant sign of desensitization. By contrast, the peak amplitude and the area of the DHβE-sensitive component did not change significantly under the same experimental conditions ($n = 4$).

**Current-Voltage Relationship of Postsynaptic Nicotinic Currents in Layer 5 Pyramidal Neurons**

In order to characterize the voltage dependence of the ionic current through nAChRs in pyramidal neurons, we determined the current–voltage relationship and the reversal potential of nicotinic membrane currents. We applied ACh by short (50 ms, $n = 7$) and long (200 ms, $n = 5$) pressure pulses and measured the amplitude of the induced currents at different holding potentials (Fig. 8A). The amplitude measurements were performed at 2 different time points, with one point...
corresponding to the peak of the initial fast component recorded at a holding potential of −70 mV (Fig. 8A, Fast). Because the presence of the initial peak amplitude was independent of the duration of the ACh pulse, its value could be determined in all neurons investigated (n = 12). The second time point was set at a latency of at least 200 ms after the onset of the current response in order to obtain values for the slow component (Fig. 8A, Slow). Because the slow current component was more prominent following ACh application with long pulses, these measurements were performed only in cases, when ACh was applied with a pressure pulse of 200 ms in duration (Fig. 8A, n = 5). The diagram in Figure 8B displays the determination of the reversal potential of the fast component. The initial amplitudes (mean ± SD) were plotted as a function of the holding potential. The dashed line represents the linear regression of the data between −80 mV and 0 mV; the interception of this line with the voltage axis revealed a reversal potential of 5.9 mV. The amplitudes of the fast current component measured at holding potentials of 20 and 40 mV deviated from the linear regression and the mean amplitudes of these currents were not significantly different from zero (20 mV: P = 0.8; 40 mV: P = 0.3, one-sample t-test). This observation reflects the well-known inward rectification of nicotinic membrane currents (Haghighi and Cooper 2000; Christophe et al. 2002). Figure 8C shows the current-voltage relationship of the slow current component. At membrane potentials close to and positive to −30 mV, the ACh-induced slow current components were very small. Their mean values were not significantly different from zero (−30 mV: P = 0.8; −10 mV: P = 0.5; 0 mV: P = 0.4; 20 mV: P = 0.5, one-sample t-test). Therefore, we estimated a reversal potential of the slow component by linear regression of the values between −80 and −50 mV and it was found to be −12 mV.

Discussion

This study shows that layer 5 pyramidal neurons of the rat neocortex express functional postsynaptic nicotinic AChRs. Following inactivation of muscarinic AChRs by atropine and blockade of synaptic transmission by TTX and glutamate receptor antagonists as well as GABA<sub>α</sub> receptor antagonists, more than two thirds of the neurons investigated responded to the local application of ACh with membrane depolarizations consisting of a fast and a slow component. These depolarizations, which sometimes were large enough to evoke action potentials, were generated by membrane inward currents composed of an initial fast component and a slow component. These ACh-induced currents were completely and reversibly blocked by coapplication of the subtype selective nicotinic receptor antagonists MLA (≤10 nM) and DHβE (1 μM) (Berg and Conroy 2002; Raggenbass and Bertrand 2002; Hogg et al. 2003). The reversal potential of the fast component was found to be 5.9 mV suggesting that the activated ion channels were permeable for cations (Karlin 2002). Due to the small current amplitudes at holding potentials positive to −30 mV and the increased current baseline oscillations at these potentials, we could provide only an estimation of the reversal potential of the slow component. Considering the variability of the amplitudes, the value determined (−12 mV) does not argue against a membrane current mediated by cation-permeable channels.

The observation that about 30% of the neurons tested did not react to the ACh application suggests an inhomogeneous population of neurons, despite the fact that all of the neurons analyzed were morphologically and electrophysiologically identified as regular spiking pyramidal cells. A small fraction of regular spiking neurons within the neocortex are GABAergic interneurons (Kawaguchi 1993). Because they can be distinguished from pyramidal cells by the morphology of their somata, it is unlikely that we encountered a large fraction of ACh-insensitive nonpyramidal-shaped regular spiking neurons. Thus, similar to layer 5 inhibitory interneurons (Xiang et al. 1998), layer 5 pyramidal cells may display a differential sensitivity to the activation of nAChRs. However, we cannot completely exclude other possibilities, which might have contributed to the lack of effect of ACh in about 30% of the cells. These possibilities include a very fast desensitization of nAChRs in the nonresponding neurons or different release properties of the application pipettes. In addition, because the experiments were conducted using juvenile rats (P15–P25), developmental factors might have been responsible for the lack of effect of ACh in 30% of the neurons registered and for the large interneuronal variability of the initial current amplitude (see Table 1).

With the exception of MLA (at concentrations ≤10 nM), there exist no other highly selective nicotinic receptor antagonists which would allow an unambiguous differentiation of nAChRs composed of different subunits during electrophysiological experiments in slice preparations. In order to overcome this problem to some extent, Zoli et al. analyzed the properties of β2 nAChR subunit mutant mice and provided a very useful functional classification of neuronal nAChRs (Gotti et al. 2007; Zoli et al. 1998). By means of autoradiography, receptor binding analysis and patch-clamp recording in slice preparations, they identified 4 different types of functional nAChRs. Type-1 receptors are characterized by a high affinity for α-bungarotoxin and low affinities for nicotine and ACh. Mice lacking the α<sup>7</sup> subunit of the nAChR do not express type-1 receptors (Orr-Urtreger et al. 1997), suggesting that these binding sites are homo-pentameric receptors composed of α<sup>7</sup> subunits (Zoli et al. 1998). These α<sup>7</sup>-like nAChRs are selectively blocked by MLA (≤10 nM) (Berg and Conroy 2002; Raggenbass and Bertrand 2002). They display a very fast desensitization and are localized predominantly in neocortical and limbic structures of the brain (Zoli et al. 1998). Because the initial fast component of the ACh-induced inward current, which we observed in pyramidal cells in the presence of atropine, were reversibly blocked by 10 nM MLA and showed a fast desensitization, we conclude that this component (the MLA-sensitive component, see Figs 4 and 5) was mediated by nAChRs consisting of α<sup>7</sup> subunits.

Type-2 nAChRs in the classification provided by Zoli et al. (1998) are endowed with a high affinity for nicotine, a low affinity for ACh and disappear in mice lacking the α<sup>4</sup> subunit (Marubio et al. 1999; Ross et al. 2000) and/or the β2 subunit of the nAChR (Zoli et al. 1998). These α<sup>4</sup>β<sup>2</sup>-like receptor subtypes are found throughout the brain and represent the vast majority of nAChRs in the CNS (Cordero-Erausquin et al. 2000). They are insensitive to MLA and are selectively antagonized by DHβE (≤10 μM) (Zoli et al. 1998; Raggenbass and Bertrand 2002). Because the slow component of the membrane current induced in pyramidal cells by local application of ACh was reversibly blocked by DHβE (1 μM), we conclude that this component (the DHβE-sensitive component, see Figs 4 and 5) was mediated by the α<sup>4</sup>β<sup>2</sup>-like subtype of the nAChR.
The type-3 and type-4 nAChRs classified by Zoli et al. (1998) are hetero-pentameric complexes in which the β4 subunit combines with different α subunits. They are MLA-insensitive and very scarcely expressed in the neocortex. As we could block the ACh-induced current response completely by coapplication of MLA and DHβE in all of the neurons tested, it seems unlikely that these receptor types contributed to the membrane currents we observed in this study.

Following pharmacological suppression of muscarinic mechanisms, application of ACh into the vicinity of pyramidal neurons can influence the activity of these cells in at least 4 different ways. 1) ACh can activate nAChRs present at somatodendritic sites of neurons presynaptic to the pyramidal neuron under investigation (McCormick and Prince 1986; Nicoll et al. 1996; Porter et al. 1999; Alkondon et al. 2000; Christophe et al. 2002; Klaassen et al. 2006; Couey et al. 2007). The consequence would be an excitation or an inhibition of the pyramidal cell mediated either by an excitatory or an inhibitory neurotransmitter. McCormick and Prince first showed that inhibition of pyramidal neurons by activation of nAChRs on GABAergic interneurons is a common mechanism in the neocortex (McCormick and Prince 1986). Our data support this finding. Following blockade of muscarinic AChRs, ACh induced an initial depolarization often followed by barrages of synaptic potentials, which could be blocked by the combined application of TTX, glutamate receptor antagonists, and GABA_A receptor antagonists. Therefore, these barrages most probably reflect the activation of nAChRs on interneurons presynaptic to the pyramidal neuron recorded. Because we were interested in postsynaptic ACh effects, we pharmacologically suppressed synaptic transmission from presynaptic neurons to the pyramidal cell under investigation.

The second way in which locally applied ACh can influence the activity of a pyramidal cell is activation of nAChRs located on axons of presynaptic neurons. Binding of ACh to these so-called preterminal nAChRs (Wonnacott 1997; Dani 2001) leads to a depolarization of the axonal membrane and therefore to an increase in excitability of the axon. The result would be a facilitation of transmitter release. In our experiments, we prevented this mechanism by application of TTX.

All nAChRs are cation-permeable ionotropic receptors with a significant permeability for calcium ions (Karlin 2002). Thus, activation of nAChRs located directly on presynaptic boutons results in a calcium influx into the bouton and subsequent transmitter release from the terminal. This mechanism represents a third possibility to influence the activity of a pyramidal neuron by local application of ACh (Wonnacott 1997; Dani 2001). We suppressed this mechanism by blocking the receptors for the most abundant neurotransmitters (glutamate receptors and GABA_A receptors) in the neocortex. However, we cannot exclude the small possibility that nAChRs are located on presynaptic boutons of neurons which do not release glutamate or GABA.

The fourth way to influence pyramidal neurons by ACh application is to activate postsynaptic nAChRs expressed by these neurons themselves. Because we excluded to a far extent all other possibilities discussed above, we conclude that the membrane depolarizations and membrane inward currents observed in pyramidal neurons following local application of ACh were mediated by postsynaptic nAChRs of the α7-like and α4β2-like subtype. This conclusion is supported by the fact that it was possible to determine the reversal potential of the ACh-induced currents by changing the holding potential of the voltage-clamped neuron. The reversal potential we have found is in agreement with other studies on ACh-induced currents in neocortical neurons (Porter et al. 1999; Christophe et al. 2002) and other cells (Haghighi and Cooper 1998; Haghighi and Cooper 2000). Furthermore, we showed that the initial fast current component displayed the well-known inward rectification of nicotinic currents (Haghighi and Cooper 1998; Christophe et al. 2002).

The postsynaptic nicotinic membrane currents recorded from neocortical pyramidal neurons following application of ACh to the cells’ somata resembled the currents described in interneurons of the hippocampus (Alkondon and Albuquerque 1993) as well as of the rodent and human neocortex (Porter et al. 1999; Alkondon et al. 2000; Christophe et al. 2002). All of these ACh-evoked membrane currents consisted of 2 components overlapping each other. As we could show in this study, the fast component is most likely mediated by the α7-like subtype of the nAChR and in most cases displays a fast rise time and a relatively fast decay. The slow component is most likely mediated by the α4β2-like subtype of the nAChR and is characterized by a slow time course of the rise and the decay time. Both current components are subject to desensitization to varying extents. The physiological significance of nAChR desensitization is not clear. At a cholinergic synapse, ACh (ca. 1 mM) is rapidly delivered to the receptors and removed within milliseconds from the receptors (Dani 2001). Thus, receptor desensitization does not seem to be of physiological importance. However, desensitization can be considered as a regulatory mechanism for the control of synaptic efficacy. It has been proposed that the number of active nAChRs is determined by controlled desensitization (Quick and Lester 2002; Guo and Lester 2007). This may open the possibility for modulation of nicotinic currents by desensitization.

**Functional Significance of Postsynaptic nAChRs Expressed by Neocortical Pyramidal Neurons**

Within the neocortex, functional nAChRs have been demonstrated to be expressed on the somata of GABAergic inhibitory interneurons (McCormick and Prince 1986; Nicoll et al. 1996; Xiang et al. 1998; Porter et al. 1999; Alkondon et al. 2000; Christophe et al. 2002; Couey et al. 2007), on axons of thalamocortical afferents (Kawai et al. 2007), and on presynaptic terminals of inhibitory and excitatory afferents projecting onto pyramidal cells (Vidal and Changeux 1993; Gil et al. 1997; Wonnacott 1997). However, the expression of nAChRs seems to be restricted to certain neuronal subtypes in different layers (Xiang et al. 1998; Porter et al. 1999) and to certain neocortical afferents (Gil et al. 1997), suggesting a very complex cholinergic network within the neocortex with highly specific functions. One possible function of postsynaptic nAChRs on pyramidal neurons might be the generation of nicotinic EPSPs. Two studies observed such EPSPs in neocortical pyramidal cells (Roerig et al. 1997; Chu et al. 2000). However, the way in which cholinergic fibers contact their neocortical target neurons is still a matter of discussion. Classical cholinergic synapses have been shown to be present throughout the neocortex (Wa¨ner et al. 1984; Houser et al. 1985; Turrini et al. 2001). The majority of these synapses consists of symmetric contacts with dendritic shafts and spines as well as with cell somata (Houser et al. 1985; Turrini et al. 2001). Some of these cholinergic synapses were of the...
asymmetric type and it has been shown that α7 subunits of the nAChR are predominantly localized at such synapses (Lubin et al. 1994; Levy and Aoki 2002). On the other hand, evidence has been provided that cholinergic fibers form boutons en passant (Umbrico et al. 1994; Henny and Jones 2008). Thus, the possibility exists that ACh modulates the activity of neocortical neurons via classical synaptic transmission or volume transmission or both simultaneously (Semba 2000).

Due to the scarce evidence for nicotinic EPSPs in neocortical pyramid cells, one might speculate that ACh modulates the activity of layer 5 pyramid cells predominantly via volume transmission. In particular, α7 subunits have been localized in a so-called perisynaptic position, i.e. in the close vicinity of glutamatergic synapses but outside the postsynaptic density of the synaptic apparatus (Fabian-Fine et al. 2001; Berg and Conroy 2002; Levy and Aoki 2002). Such a location together with the calcium permeability of nAChRs suggests that ACh might facilitate or disfacilitate glutamatergic synaptic transmission in layer 5 pyramid cells via postsynaptic nAChRs. In fact, a number of studies provided evidence that activation of nAChRs influences neocortical plasticity (Aramakis and Metherate 1998; Hasselmo 2006; Levy et al. 2006; Couey et al. 2007; Rozsa et al. 2008).

**Supplementary Material**

Supplementary material can be found at: http://www.cercor.oxfordjournals.org/

**Notes**

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