Two Types of Nicotinic Receptors Mediate an Excitation of Neocortical Layer I Interneurons

ELODIE CHRISTOPHE,1 ALINE ROEBUCK,2 JOCHEN F. STAIGER,3 DANIEL J. LAVERY,2 SERGE CHARPAK,1 AND ETIENNE AUDINAT1

1Laboratoire de Neurophysiologie, Institut National de la Santé et de la Recherche Médicale EPI 0002, ESPCI, 75231 Paris Cedex 5, France; 2GlaxoSmithKline SA, Institut de Biologie Cellulaire et de Morphologie, 1005 Lausanne, Switzerland; and 3Heinrich-Heine University, C & O Vogt-Institute for Brain Research, D-40001 Dusseldorf, Germany

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Christophe, Elodie, Aline Roebuck, Jochen F. Staiger, Daniel J. Lavery, Serge Charpak, and Etienne Audinat. Two types of nicotinic receptors mediate an excitation of neocortical layer I interneurons. J Neurophysiol 88: 1318–1327, 2002; 10.1152/jn.00199.2002. Nicotinic acetylcholine receptors are widely expressed in the neocortex but their functional roles remain largely unknown. Here we investigated the effect of nicotinic receptor activation on interneurons of layer I, which contains a high density of cholinergic fiber terminals. Ninety-seven of 101 neurons recorded in whole cell configuration in rat acute slices were excited by local pressure application of nicotinic agonists, acetylcholine (500 µM), 1,1-dimethyl-4-phenyl-piperazinium (500 µM) or choline (10 mM). Biocytin labeling confirmed that our sample included different morphological types of layer I interneurons. The responses to nicotinic agonists persisted in presence of glutamate and muscarinic receptor antagonists and on further addition of Cd2+ or tetrodotoxin, indicating that they were mediated by direct activation of postsynaptic nicotinic receptors. The kinetics of the currents and their sensitivity to nicotinic receptor antagonists, methyllycaconitine (1–10 nM) or dihydro-β-erythroidine (500 nM), suggested that early and late components of the responses were mediated by α7 and non-α7 types of receptors. Both components had inwardly rectifying I-V curves, which differed when intracellular spermine was omitted. Single-cell RT-PCR experiments identified α4, α7, and β2 as the predominantly expressed mRNAs, suggesting that the receptors consisted of α7 homomers and α4β2 heteromers. Finally, selective excitation of layer I interneurons through activation of their nicotinic receptors resulted in a tetrodotoxin-sensitive increase of inhibitory synaptic currents recorded in nonpyramidal cells but not in pyramidal cells of layer II/III. These results suggest that acetylcholine released in layer I may induce a disinhibition of the cortical network through activation of nicotinic receptors expressed by layer I interneurons.

INTRODUCTION

Nicotinic receptors (nAChRs) are implicated in a variety of functions of the mammalian cerebral cortex, as for instance memory formation (Granot et al. 1995) and regulation of cerebral blood flow (Gitelman and Prohovnik 1992; Uchida et al. 1997). Some of these functions might be compromised in normal aging but also in Alzheimer’s disease, which has been associated, at least in part, with a decrease of the level of nAChRs and a loss of cholinergic innervation (James and Nordberg 1995; Whitehouse et al. 1986). It seems thus essential to understand the molecular and cellular basis of the effects mediated by the activation of nAChRs in cortical circuits.

The major cholinergic innervation of the rat neocortex stems from the CH4 cell group of the nucleus basalis of Meynert (nbM) (Everitt et al. 1988; Mesulam et al. 1983). These fibers follow different trajectories between the nbM and the cerebral cortex (Selden et al. 1998). They enter the cortex and initially run within layer VI to terminate mainly in layers V and I (Kristt et al. 1985). Indeed, the layer I has been showed to contain the highest laminar densities of ACh axons and varicosities (Mechawar et al. 2000) and could be then a major site of action of acetylcholine (ACh). The targets of the cholinergic fibers in this layer could be the apical dendrites of pyramidal and bipolar cells from layer II to V that extend terminal tufts up to layer I. However, the release of ACh in layer I could also lead to the activation of neurons with somata located in layer I. There have been only few functional studies of layer I neurons (Hestrin and Armstrong 1996; Martin et al. 1989; Zhou and Hablitz 1996), which are nonpyramidal cells (Prieto et al. 1994) found in low density beneath the pial surface. They almost all stain for glutamate decarboxylase or GABA and are thus probably GABAergic interneurons (Gabbott and Bacon 1997; Li and Schwark 1994; Prieto et al. 1994; Winer and Larue 1989).

In the present study, we tested the sensitivity of layer I interneurons to local application of nAChR agonists. We characterized the nicotinic responses of these interneurons by means of whole cell recordings, pharmacological tools, and single-cell RT-PCR. The results show that, despite their different morphologies, almost all layer I interneurons respond to nAChR agonists, via the activation of both α7 and non-α7 subtypes of nAChRs.

METHODS

Slice preparation

Coronal and parasagittal sections (300 µm thick) from motor, somatosensory, and parietal associative cerebral cortex of 14- to 25-day-old Wistar rats were prepared as previously described (Cauli et al. 1997)
using a vibroslicer (Leica, Wetzlar, Germany). Similar results were obtained with all types of slices used, and thus results were pooled together. Slices were incubated at room temperature (20–25°C) in a recording solution containing (in mM) 121 NaCl, 2.5 KCl, 1.25 NaCl, 1 MgCl$_2$, 10 EGTA, 1 CaCl$_2$, 10 phosphocreatine, 4 ATP-K$_2$, and 5 pyruvate (pH 7.2, 325 mosM) and bubbled with a mixture of 95% O$_2$-5% CO$_2$.

**Recordings**

For the recordings, slices were transferred to a chamber perfused at 2 ml/min with the extracellular solution at room temperature. We selected cortical neurons from layers I–III under visual control using an upright microscope equipped with Nomarski differential interference contrast optics and a water-immersion objective. Patch pipettes (3–5 MΩ) were pulled from borosilicate glass (Harvard Apparatus LTD, Kent, UK) and were usually filled with 10 μl of intracellular solution containing (in mM) 130 K-glucuronate, 10 HEPES, 4 NaCl, 1 MgCl$_2$, 10 phosphocreatine, 0.3 GTP, and 4 ATP-K$_2$ (pH = 7.2, 295 mosM). The current-voltage relationships of the responses induced by nicotinic receptor agonists in layer I cells and the inhibitory postsynaptic currents in layers II/III neurons were recorded using an intracellular solution containing (in mM) 130 K-glucuronate, 10 HEPES, 4 NaCl, 1 MgCl$_2$, 10 phosphocreatine, 0.3 GTP, and 4 ATP-K$_2$ (pH = 7.2). Spermine (100 μM) was added to this intracellular solution when needed. Whole cell recordings in voltage-clamp mode or fast current-clamp mode were obtained using a patch-clamp amplifier (Axopatch 200A, Axon Instruments, Foster City, CA), and all membrane potential values were corrected for a junction potential of ~11 mV. The series resistance was not compensated but monitored throughout the experiments. Signals were filtered at 1–5 kHz, digitized at 10–20 kHz, and analyzed off-line with Acquis-1 software (G. Sadoc, Gif/Yvette, France). In current-clamp recordings, the input resistance was measured in response to a ~25 pA pulse.

Cholinergic agonists were prepared in a solution containing (in mM): 135 NaCl, 5.4 KCl, 1.8 CaCl$_2$, 1 MgCl$_2$, 10 HEPES, and 30 glucose (pH 7.4, 330 mosM) and were applied by pressure (150 ms, 100 μPa) from a patch pipette (2–3 MΩ). This application pipette was positioned approximately 50 μm from the soma of the recorded cells so that the three tested agonists 1,1-dimethyl-4-phenyl-piperazinium (DMPP, 500 μM), ACh (500 μM), and choline (10 mM) induced membrane currents with a relatively fast rising time of 47 ± 23 ms (n = 12), 41 ± 23 ms (n = 56), and 23 ± 15 ms (n = 25), respectively. Antagonists were added directly to the bathing solution. In the case of atropine, relatively high concentrations of this competitive antagonist were used (10–100 μM) to ensure a complete block of muscarinic receptors during local applications of high concentrations of ACh. Although these high concentrations of atropine occasionally decreased the peak amplitude of the responses (Zwart and Vijeerberg 1997), it did not change the relative contribution of the two components of the nicotinic responses (see RESULTS). All reported values are expressed as the means ± SD. Between-group comparisons were performed using a Mann-Whitney nonparametric test or an unpaired two-tailed Welch t-test that does not assume equal SDs. All the chemical products were from Sigma (St Louis, MO) except CNQX, N-2-amino-5-phosphonoveric acid (n-APV), and bicuculline, which were from Tocris Cookson (Bristol, UK).

**RT-PCR single cell**

Cytoplasm harvesting and reverse transcription were performed as previously described (Lambolez et al. 1992). Briefly, patch pipettes were filled with 8 μl of solution containing (in mM) 144 K-glucuronate, 3 MgCl$_2$, 10 HEPES, and 0.2 EGTA (pH 7.2, 295 mosM). After the recording, the content of the cell was aspirated into the pipette and expelled in a test tube for reverse transcription reactions. The usual volume recovered was approximately 6 μl. This 6 μl was brought to 10 μl with the following components at final concentrations as indicated: 5 μM of hexamer random primers, 0.5 mM of each of the four deoxyribonucleotide triphosphates, 1.2 mM of MgCl$_2$, 2 mM of Tris pH 8, 10 mM of dithiothreitol, 20 U of ribonuclease inhibitor (Promega), and 200 U of Moloney murine leukemia virus reverse transcriptase (Promega). The resulting mix was incubated overnight at 37°C and then frozen at ~20°C until PCR amplification.

Two steps of multiplex PCR were run to amplify the neuronal nicotinic receptor subunits (α2, α3, α4, α5, α6, α7, β2, β3, and β4). The cDNAs present in the reverse transcription reaction were first amplified in a final volume of 50 μl with 0.2 μM of each of the nine primer pairs described previously (Porter et al. 1999), 0.2 mM of each of the deoxyribonucleotides triphosphate, 1–2 U of Taq polymerase (as recommended by supplier), and the buffer containing (in mM) 50 Tris (pH 8.9), 50 KCl, and 1.5 MgCl$_2$. Twenty PCR cycles (1 min at 94°C, 1 min 30 s at 55°C, 1 min 30 s at 73°C) were then performed, with an initial elongation period of 5 min at 94°C and a final period of 10 min at 73°C. Of this reaction, 2 μl was then used as template for the second, gene-specific rounds of PCR. Each cDNA was individually amplified during 35 cycles using the following sets of nested primers [5′ to 3′ coordinates according to sequence entries cited by Porter et al. (1999)]: α2 (192–211 and 375–393), α3 (71–91 and 217–236), α4 (392–411 and 568–587), α5 (1246–1265 and 1427–1447), α6 (156–172 and 416–435), α7 (102–121 and 364–384), β2 (224–243 and 461–480), β3 (264–283 and 507–526), and β4 (385–404 and 648–667). The predicted sizes of the PCR products were: α2 (201 bp), α3 (165 bp), α4 (195 bp), α5 (201 bp), α6 (282 bp), α7 (282 bp), β2 (256 bp), β3 (262 bp), and β4 (282 bp).

Each PCR reaction (15 μl of the 50 μl reaction) was run on a 1.6% agarose gel stained with ethidium bromide, using a 100-bp DNA ladder molecular weight marker. The efficiency of the RT-multiplex PCR protocol was tested on 500 pg total RNA from rat brain for each primer pair. Criteria for inclusion were results in which the positive control reaction (500 pg total rat brain RNA plus reverse transcriptase) demonstrated a single major band of correct size, while the negative control reaction (RNA, no reverse transcriptase) generated no detectable products other than primer dimers.

**Histology**

Biocytin was added to the intracellular solution (2 mg/ml), and the slices containing biocytin-filled cells were fixed overnight in a solution of 4% paraformaldehyde in 0.1 M phosphate buffer (PB, pH = 7.4) or in phosphate-buffered saline (PBS, pH = 7.4) at 4°C. After extensive rinsing with PB, slices were incubated with a cryoprotectant solution (25% saccharose, 10% glycerol, in 0.01 M PB) for 1 h and then freeze-thawed three times over liquid nitrogen. After rinsing with PB, an intermediate blocking step of endogenous peroxidase activity was performed, with H$_2$O$_2$ (1% in PB) was introduced. Subsequently, the slices were incubated overnight with ABC (Vector, Burlingame, CA). After several rinses with PB, 0.5 mg/ml 3,3′diaminobenzidine (Sigma) was preincubated for 10 min. Then the peroxidase reaction was started by adding H$_2$O$_2$ (in PB) to a final concentration of 0.01% and stopped by rinsing with PB. The reaction product was intensified with OsO$_4$ (14% in PB). After being washed with distilled water, slices were mounted in Vectashield solution (Vector). Coverslips were sealed with fingernail polish for storage. Morphology of filled neurons was reconstructed with the Neurolucida software (MicroBrightfield, Colchester, VT). The lengths of dendrites and axons were measured using the Neuroexplorer software package (MicroBrightfield). Digital pictures were acquired with a CCD camera.

**RESULTS**

**Morphologies and firing behaviors of layer I neocortical interneurons**

Layer I interneurons were visually identified in acute slices of the neocortex by means of infrared microscopy. Thirteen of
the recorded neurons were stained by biocytin injection (see METHODS). These interneurons were morphologically diverse as shown previously by others (Bradford et al. 1977; Hestrin and Armstrong 1996; Zhou and Hablitz 1996). In general, their somata were variable in shape and size (Fig. 1, A and B) with a largest area that ranged from 58.3 to 161 μm² (n = 11). Their dendrites were smooth (n = 11) or sparsely spiny (n = 2) and mostly confined to layer I with the exception of one cell, which had a branch descending into layers II/III.

The axonal arborizations of layer I interneurons could be classified in two types. The first type of interneurons (n = 5) had axons confined to layer I (Fig. 1, A, C, and D). Three of these cells had a multipolar dendritic arborization with a radius of 98.3 ± 22.5 μm around the soma and a short axon of a total length of 410.6 ± 231.8 μm corresponding to the previously described neurogliaform interneurons (Hestrin and Armstrong 1996). The two other cells had horizontal axonal arborization that extended up to 420 μm from the soma with a total length of 2.0 ± 0.6 mm. They possessed a multipolar dendritic arborization. The second type (n = 6) showed axon collaterals leaving layer I to extend mainly within layers II/III but also, in one case, to reach layer V (Fig. 1E). The axonal arbor of these neurons had a total length on the average of 2.8 ± 2.7 mm (n = 6). Their multipolar dendritic arborization, with three to nine primary dendrites, showed a radius of 230.0 ± 27.4 μm around the soma (n = 6). Finally, we also observed a layer I interneuron with two axons, one descending in layers II/III and one extending horizontally in layer I (Fig. 1B). A similar case has been previously reported (Bradford et al. 1977).

Despite these different morphologies, layer I interneurons did not differ markedly from each other in their intrinsic electrical membrane properties (data not shown). The resting membrane potential and the input resistance of the neurons stained with biocytin were -75 ± 7 mV and 405 ± 142 MΩ (n = 15), respectively. The first action potential elicited by a depolarizing step of current had an amplitude of 74 ± 5 mV and a duration of 1.2 ± 0.2 ms (n = 15). The afterhyperpolarization (AHP) following the first spike had an amplitude of 6 ± 4 mV (n = 15). The firing behavior of all tested layer I interneurons resembled that of nonregular spiking nonpyramidal cells (Cauli et al. 1997; Kawaguchi 1995) and was characterized by a marked frequency adaptation. The instantaneous
action potential frequency was reduced by 70.5 ± 11.5% during a response to a depolarizing pulse of 800 ms that induced an initial instantaneous frequency near 100 Hz (Cauli et al. 2000).

Postsynaptic responsiveness to nicotinic agonists: a common property of layer I interneurons

Ninety-seven of the 101 tested neurons presented similar responses to brief local applications of nicotinic receptor agonists (see METHODS). These responses were obtained in the presence of the AMPA/kainate receptor antagonist CNQX (12.5 μM), the NMDA receptor antagonist d-APV (25 μM), and the muscarinic receptor antagonist atropine (10–100 μM) in the bathing solution together with the GABA_A receptor antagonist bicuculline (20 μM; n = 9) or with tetrodotoxin (0.5 μM; n = 16) or cadmium (10 μM; n = 3). These results indicate that the agonist-induced responses were mainly due to the activation of postsynaptic cholinergic receptors located on the soma and the dendrites of layer I interneurons. During recordings in current-clamp mode in the presence of CNQX, d-APV, and atropine, the local application of nicotine composed of 7 subunits (Alkondon et al. 1996; Zoli et al. 1998). Bath application of 1–10 nM MLA completely blocked the response to 10 mM choline (Fig. 3A) and strongly reduced that to 500 μM ACh (Fig. 3B). In the presence of MLA, the peak amplitude of the remaining responses represented 8.4 ± 4.8, 2.5 ± 2.1, and 9.0 ± 1.4% of that of the control response to ACh (n = 4), choline (n = 5), and DMPP (n = 4), respectively (Fig. 3C). The effects of the antagonist, especially for ACh, were less marked when the integral of the responses was taken into account. In the presence of MLA, the integral of the remaining response was 40.9 ± 6.8, 10.9 ± 9.7, and 12.2 ± 11.8% of that of the initial response to ACh (n = 4), choline (n = 5), and DMPP (n = 4), respectively (Fig. 3C).

The remaining response to ACh, in the presence of 10 nM MLA, showed relatively slow kinetics (Fig. 3B). The time to peak of ACh response increased from 19.4 ± 16.4 to 1142.5 ± 512.1 ms on application of MLA (P < 0.05, Mann-Whitney test; n = 4). These slow responses were comparable to that mediated by non-α7 subtypes of nAChRs in bipolar interneurons of layer II–V recorded in similar conditions (Porter et al. 1999). Therefore we tested the action of dihydro-β-erythroidine (DHβE), a nonselective antagonist of nicotinic receptors, which appears, however, to have a higher affinity for non-α7 subtype of nACh (Alkondon and Albuquerque 1993). Bath application of DHβE (500 nM) diminished the amplitude of both the early and late components of the responses to 10 mM choline (Fig. 3D) and to 500 μM ACh (Fig. 3E). The peak amplitude of the remaining responses represented 76.5 ± 12.2, 58.8 ± 5.4, and 64.2 ± 9.6% of control responses to ACh (n = 10), choline (n = 5), and DMPP (n = 6), respectively (Fig. 3F). When the traces were normalized to the initial peak amplitude, we observed that DHβE did not modify the kinetics of the choline responses (Fig. 3D, bottom) but shortened the duration of those to ACh (Fig. 3E, bottom). Accordingly, DHβE decreased more the integral of the currents induced by ACh than those induced by choline and DMPP. The current integral remaining under DHβE corresponded to 41.5 ± 16, 70.4 ± 9.2, and 60.4 ± 12.3% of that of the initial response to ACh (n = 10), choline (n = 5), and DMPP (n = 6), respectively (Fig. 3F). Thus DHβE weakly antagonized choline responses, entirely mediated by α7 subtypes of nAChR but more potently antagonized the late component of ACh responses probably mediated by both α7 and non-α7 subtypes of nAChRs.

Layer I interneuronal responses are mediated by α7 and non-α7 subtypes of nAChRs

To test for the expression of different types of nicotinic receptors by layer I interneurons, we first tested the effect of methyllycaconitine (MLA), a selective antagonist of the α7 subtype of nAChR (Alkondon and Albuquerque 1993; Palma et al. 1996; Zoli et al. 1998). Bath application of 1–10 nM MLA completely blocked the response to 10 mM choline (Fig. 3A) and strongly reduced that to 500 μM ACh (Fig. 3B). In the presence of MLA, the peak amplitude of the remaining responses represented 8.4 ± 4.8, 2.5 ± 2.1, and 9.0 ± 1.4% of that of the control response to ACh (n = 4), choline (n = 5), and DMPP (n = 4), respectively (Fig. 3C). The effects of the antagonist, especially for ACh, were less marked when the integral of the responses was taken into account. In the presence of MLA, the integral of the remaining response was 40.9 ± 6.8, 10.9 ± 9.7, and 12.2 ± 11.8% of that of the initial response to ACh (n = 4), choline (n = 5), and DMPP (n = 4), respectively (Fig. 3C).

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FIG. 2. Responses of layer I interneurons to local applications of different nicotinic receptor agonists. A and B: responses of the same interneuron to local applications of acetylcholine (ACh, 500 μM) recorded in voltage (A)- and in current-clamp (B) modes. C and D: responses of 2 layer I interneurons recorded in voltage-clamp to the nicotinic agonists 1,1-dimethyl-4-phenylpiperazinium (DMPP, 500 μM; C) and choline (10 mM; D). ↓, the beginning of the applications. The resting membrane potential of the cells in B was −70 mV and the holding potential was adjusted at −71 mV in the voltage-clamp recordings (A, C, and D). All responses were obtained in the presence of 6-cyano-7-nitroquinoxaline-2,3-dione (CNQX, 12.5 μM), D-2-amino-5-phosphonovaleric acid (D-APV, 25 μM), and atropine (10 μM) in the extracellular bathing solution.
nAChRs. The co-application of the two antagonists MLA and DH\(\beta\)E completely blocked the responses to ACh \(\left(10^{-4}\right)\); Fig. 3, B and E).

Altogether these results indicated that the responses to ACh applications in layer I interneurons were due to the activation of two types of nAChRs, \(\alpha7\) subtypes mediating most of the early component and non-\(\alpha7\) subtypes contributing mainly to the late component.
Rectification of the I-V curve of the nicotinic responses of layer I interneurons

To further characterize the two components of the responses induced by the nicotinic receptor agonists, we analyzed their current-voltage relationship. We measured the amplitude at the peak and 800–1,000 ms after the peak of the currents induced by brief applications of ACh (500 μM) at various membrane potentials in the presence or absence of intracellular spermine (100 μM). Indeed, spermine is a ubiquitous intracellular polypeptide known to block, among other channels, nicotinic receptors in a voltage-dependent manner (Haghighi and Cooper 1998). When spermine was included in the recording pipette, the I-V curves of the early and late components of the nicotinic responses were strongly inwardly rectifying. Figure 4A shows an example of nicotinic responses of the same interneuron recorded successively with two different patch pipettes containing an intracellular solution (see METHODS), first without (Fig. 4A, left), then with spermine (Fig. 4A, right). For both the early and late components, the amplitude of the currents recorded at negative potentials did not differ between both conditions and the reversal potential was near +10 mV. The amplitude of the outward currents obtained at positive potentials for the early but not for the late component was, however, larger when spermine was omitted (Fig. 4A). Figure 4B shows the normalized I-V relationships for the early (Fig. 4B, top) and late (Fig. 4B, bottom) components of ACh responses recorded in different layer I interneurons without (○, ■; n = 6) or with (●, □; n = 16) intracellular spermine. The I-V curve of the early component showed a more pronounced inward rectification in the presence of spermine and there was a significant difference between the amplitude of the outward currents obtained with or without spermine at +30 mV (P < 0.001) and at +50 mV (P = 0.006). The I-V curves of the late component did not differ significantly, even at positive potentials, when spermine was included or not in the intracellular solution. To confirm that the late component was less sensitive to the washout of intracellular spermine, we analyzed the I-V curve of this component isolated pharmacologically. The slow currents, induced by 500 μM ACh in the presence of MLA (10 nM) and recorded after an extended dialysis in the whole cell configuration with a solution without spermine, had an I-V curve with a strong inward rectification with no outward currents detected at positive membrane potentials (n = 2; data not shown).

These observations further confirmed the presence, on layer I interneurons, of two types of nicotinic receptors that have probably different affinities for spermine and are responsible for the early and late components of the responses.

Nicotinic receptor subunit mRNAs expressed by layer I interneurons

The nicotinic receptor subunits predominantly expressed in the rat neocortex are α3, α4, α5, α7, and β2. To identify which of these subunits were expressed in layer I interneurons and could therefore be responsible of the early and late components of the responses induced by nicotinic agonists, 26 responsive interneurons were analyzed by single-cell RT-PCR (Porter et al. 1999) (see METHODS). In agreement with the pharmacological profile of the responses, mRNAs for α5 and non-α7 subunits were detected in a majority of layer I interneurons (Fig. 5, B and C). The most predominantly expressed subunit was α4 which was detected in 24 of 26 tested cells, α7 and β2 mRNAs were detected in more than half of the cases, whereas the other subunits were either found in less than 25% of the neurons (α3, α5, and α6) or in none at all (α2, β3, and β4). These results indicate that nicotinic receptors expressed in layer I interneurons are probably hetero-oligomers made of the combination of α4 and β2 subunits on the one hand and α7 homo-oligomers on the other hand.

FIG. 4. Current-voltage relationships of the nicotinic responses of layer I interneurons. A, top: currents induced at various holding potentials by local applications of ACh (500 μM) in a layer I interneuron successively recorded, 1st in the absence (left), and 2nd in the presence (right), of 100 μM spermine in the intracellular solution. Bottom: the I-V curves from the above currents measured at the peak of the responses (●, ○) and during the decay phases (●), (○) without (left) or with (right) intracellular spermine. B: normalized I-V relationships, for the early component (top) measured at the peak of the current (●, ○), and for the late component (bottom) measured between 800 and 1,200 ms after the initial peak (●, ○), when spermine was included (●, ○; n = 6) or omitted (○, ○; n = 16) in the intracellular solution.
Effect of a selective activation of layer I interneurons on deeper cortical layers

Our present results reveal that, unlike other neuronal types of the rodent neocortex (Porter et al. 1999; Xiang et al. 1998), almost all layer I interneurons express functional postsynaptic nAChRs which, when activated, can bring the membrane potential of these neurons beyond action potential threshold (see Fig. 2B). We used this common property to investigate the potential targets of layer I interneurons in deeper cortical layers. In presence of CNQX (12.5 µM), d-APV (25 µM), and atropine (100 µM), we analyzed whether local application of ACh onto layer I interneurons changed the frequency of the inhibitory postsynaptic currents (IPSCs) recorded in pyramidal and nonpyramidal cells of layer II/III (Fig. 6A). We did not observe any change in the frequency of the IPSCs recorded in pyramidal cells (n = 33). In contrast, an increase of the IPSC frequency was observed in 12 of 42 nonpyramidal cells of layer II/III in response to pressure application of ACh in layer I. The increase of IPSC frequency was blocked by perfusion of 0.5 µM tetrodotoxin (n = 2; Fig. 6B) and of 20 µM bicuculline (n = 2; Fig. 6C), indicating that the evoked IPSCs resulted from a release of GABA triggered by action potentials. The increase of IPSC frequency had a pharmacological profile similar to that of the postsynaptic currents induced nicotinic receptor agonists in layer I interneurons: it was slightly diminished by perfusion of DHβE (n = 2; Fig. 6D) and completely blocked after further addition of MLA (n = 4; Fig. 6D). Moreover, in two other interneurons, we observed that 10 nM MLA alone completely blocked the increase of IPSC frequency (Fig. 6E).

These results demonstrate that the main targets of layer I interneurons in layer II/III are nonpyramidal cells that can be inhibited when nicotinic receptors of layer I interneurons are selectively activated.

DISCUSSION

Our results show that almost all layer I interneurons can be excited by nAChR agonists. This excitation is always mediated by the activation of two types of nAChRs, which differ from each other in their kinetics, their pharmacological characteristics, and their subunit mRNA composition. Furthermore by activating selectively the nicotinic receptors of layer I interneurons, we show that the predominant role of these interneurons is to inhibit layer II/III nonpyramidal cells and thus probably to disinhibit the cortical network.
Expression of functional postsynaptic α7 and non-α7 subtypes of nAChRs in layer I interneurons

Almost all layer I interneurons recorded in the present study were responsive to nicotinic receptor agonists. This suggests that this sensitivity is a common property of all neuronal types present in this layer of the neocortex. Indeed, the morphological diversity of the responsive neurons that we characterized morphologically corresponds to the neuronal heterogeneity described so far in the layer I of mature rodents (Bradford et al. 1977; Hestrin and Armstrong 1996; Zhou and Hablitz 1996). Together with the large number of tested neurons, this makes it unlikely that a particular subtype of neuron was excluded from our study. Therefore in contrast to most neurons of deeper cortical layers (Porter et al. 1999; Vidal and Changeux 1993), it seems that all neurons of layer I express postsynaptic functional nAChRs.

Electrophysiological, pharmacological and single-cell RT-PCR analyses favor the co-existence of two different types of nicotinic receptors on layer I interneurons. ACh applications induced first a fast rising current, which contributed 90% of the total amplitude of the response and was sensitive to nanomolar concentrations of MLA, a selective antagonist of α7 nAChR subtypes (Klink et al. 2001). This fast component could be mimicked by application of choline, a selective agonist of α7 nAChR subtypes (Alkondon et al. 1997b). A second slow-rising long-lasting component representing 40% of the total current integral was more sensitive to DHβE, a nonselective antagonist of nAChRs. The presence of two components, one involving α7 subunits and one other involving non-α7 subunits is consistent with the results of single-cell RT-PCR indicating that a significant proportion of layer I neurons co-expressed α7, α4, and β2 subunit mRNAs. The fast-rising early component was thus probably due to the activation of homomeric α7 receptors while the slow component corresponded to the activation of αβ2 heteromeric receptors. We cannot totally exclude, however, other combinations such as an association of α7 with β2 (Khiroug et al. 2002; Yu and Role 1998) or, for a small proportion of layer I interneurons, the association of α5 with α4 and β2. Surprisingly the α7 and β2 mRNAs were detected in a smaller fraction of layer I interneurons than α4 mRNAs. Electrophysiological and pharmacological data favored the coexpression of both α7 and non-α7 functional receptors in almost all layer I interneurons. This apparent discrepancy was not due to detection failures because all tested nicotinic subunit mRNAs were similarly amplified from low amounts of total RNA (see METHODS). Rather it could be explained by a low stability and differential turn over of α7 and β2 mRNAs compared with α4 mRNAs.

The two components of ACh responses in layer I cells differed also in the sensitivity of their I-V curve to the washout of intracellular spermine. Both components were inwardly rectifying at positive potentials when spermine was included in the intracellular solution. Excluding spermine, however, reduced the rectification of the fast responses but had no apparent effect on the slow responses. Similar results have been observed on the α7 and non-α7 components of nicotinic responses of hippocampal interneurons recorded without added intracellular spermine (McQuiston and Madison 1999).
result was nevertheless surprising in light of the results on heterologously expressed receptors that clearly demonstrated the voltage-dependant block of α4β2 heteromeric receptors by intracellular spermine (Haghighi and Cooper 1998, 2000). The persistence of a marked rectification of the slow non-α7 component recorded with spermine-free intracellular solution could reflect the difficulty to wash out completely the intracellular polyamines. Indeed, it has been found difficult to remove entirely the intracellular block by spermine of native channels (Fakler et al. 1995; Haghighi and Cooper 1998; Rozov and Burnashev 1999), suggesting that spermine is effectively buffed in neurons (Haghighi and Cooper 1998). If true, this would also imply that spermine has a higher affinity for α4β2 heteromers than for α7 homomers or that α4β2 receptors are expressed in distal dendrites where dialysis during the whole cell recording is less efficient.

The widespread expression of functional postsynaptic nicotinic receptors in layer I of many areas of the rat neocortex is in contrast with a restricted expression of these receptors in deeper cortical layers. In layers II–V, only a small subset of GABAergic interneurons show a somato-dendritic sensitivity to nicotinic receptor agonists, and this sensitivity is due to the expression of α4, α5, and β2 subunits (Porter et al. 1999; Xiang et al. 1998). For a long time, most of the effects of nicotinic agonists in the neocortex were thought to be only of presynaptic origin. Indeed, nAChRs modulate the release of glutamate at thalamo- and cortico-cortical synapses (Gil et al. 1997; Vidal and Changeux 1993). Functional postsynaptic α7 nAChRs were recently reported in unidentified interneurons recorded in human cortical slices (Albuquerque et al. 2000). Rat layer I interneurons described here expressed both α7 and non-α7 functional postsynaptic nAChR, and, from this point of view, they resemble interneurons of stratum oriens of the CA1 region of the hippocampus that respond to ACh with a fast α7 component and a slow non-α7 component (Buhler and Dunwiddie 2001). In the hippocampus, however, many other interneuron types are sensitive to nicotinic receptor agonists (Alkondon et al. 1999; Frazier et al. 1998; McQuiston and Madison 1999; Sudweeks and Yakel 2000).

Possible functional implication of layer I neocortical interneurons

Within the neocortex, layer I contains the highest laminar densities of ACh axons and varicosities (Mechawar et al. 2000). Our results indicate that release of ACh from these fibers could excite layer I interneurons, but the consequence of such an excitation on the activity of the cortical network remained elusive in the absence of a clearly established function of layer I cells. When applying ACh locally in layer I, we observed in nonpyramidal cells of layer II–III an increase of the IPSC frequency, which was sensitive to MLA and DHβE. This effect was blocked by tetrodotoxin and therefore was probably not due to a presynaptic effect of ACh on GABAergic terminals. Rather this result suggests that the activation of nicotinic receptors of layer I interneurons induced an action potential-dependent release of GABA onto nonpyramidal cells of deeper layers. Although more indirect effects cannot be totally excluded, they seem very unlikely because these experiments were performed in the presence of antagonists of ionotropic glutamate receptors. These results provide the first physiological evidence that most neurons in layer I are inhibitory GABAergic interneurons. They also indicate that they contact preferentially layer II/III nonpyramidal neurons because we did not observe the same increase in IPSP frequency in pyramidal neurons in response to ACh application in layer I. Although the identity and projections of the nonpyramidal cells inhibited by layer I interneurons remain to be established, they are most likely other GABAergic interneurons. Thus the physiological consequence of the cholinergic activation of layer I interneurons would be a disinhibition rather than a feedforward inhibition of pyramidal cells (Albuquerque et al. 2000; Alkondon et al. 1997a). It is well known that pyramidal neurons are excited through the activation of muscarinic receptors (Halliwell 1986; McCormick and Prince 1986). By activating nAChRs of layer I interneurons, ACh released in this layer could therefore further increase the excitation of pyramidal neurons on cholineric stimulation. Despite the small number of layer I interneurons, this effect could have a significant influence on the cortical network activity because of the large number of cholinergic terminals in layer I (Mechawar et al. 2000) and of the diverse and relatively extended axonal arborization of layer I interneurons. Of course this does not preclude other effects of ACh released in deeper layers where this neurotransmitter excites interneurons via nicotinic receptors that in turn inhibit pyramidal neurons (Porter et al. 1999).

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