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Acetylcholine receptor extracellular domain determines sensitivity to nicotine-induced inactivation

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Abstract

We have shown previously that chronic exposure to submicromolar concentrations of nicotine permanently inactivates α4β2 and α7 neuronal nicotinic acetylcholine receptors while α3β2 acetylcholine receptors are resistant to inactivation. Phosphorylation of the large cytoplasmic domain has been proposed to mediate functional inactivation. Chimeric subunits consisting of human α4 sequence from their N-terminus to either the beginning of the first transmembrane domain or the large cytoplasmic domain and α3 sequences thereafter formed acetylcholine receptors with β2 subunits which were as susceptible to nicotine-induced inactivation as wild-type α4 acetylcholine receptors. The converse chimeras, containing the N-terminal parts of the α3 subunit and the C-terminal parts of the α4 subunit, formed acetylcholine receptors with β2 subunits which were as resistant to nicotine-induced inactivation as wild-type α3β2 acetylcholine receptors. Thus, inactivation of acetylcholine receptors produced by chronic exposure to nicotine results primarily from effects of the agonist on the extracellular and transmembrane domains of the α subunit.

Keywords: Nicotinic acetylcholine receptor; Nicotine

1. Introduction

Nicotine, acting at neuronal nicotinic acetylcholine receptors, is the primary component of tobacco that drives its habitual use (Benowitz et al., 1990). Nicotinic agonists being developed as drugs to treat Alzheimer's disease, Parkinson's disease or chronic pain (Bannon et al., 1998; Holladay et al., 1997) may have some of the same effects on acetylcholine receptors as does chronic exposure to nicotine. Along with the synchronized activation of acetylcholine receptors by a rapid bolus of nicotine that results from smoking a cigarette, chronic nicotine exposure differentially affects both the amount and function of neuronal acetylcholine receptor subtypes. Acetylcholine receptors containing β2 subunits are especially important in developing dependence on nicotine, as shown by experiments with β2 knockout mice (Picciotto et al., 1998). The number of high affinity nicotine binding sites, especially α4β2 acetylcholine receptors, is increased up to fourfold in the brains of tobacco smokers and animals chronically treated with nicotine (Wonnacott, 1990; Collins and Marks, 1996; Flores et al., 1997; Perry et al., 1999). In addition, chicken α4β2 acetylcholine receptors expressed in *Xenopus* oocytes or in a permanently transfected cell line doubled in amount when chronically exposed to nicotine (Peng et al., 1994). The EC_{50} for upregulation was 0.2 μM, essentially equal to the concentration of nicotine typically found in the serum of smokers (Benowitz et al., 1990). Chronic exposure to 10 μM nicotine not only reversibly desensitized these acetylcholine receptors, but also permanently inactivated many of them (Peng et al., 1994). Similarly, human α4β2 acetylcholine receptors in a permanently transfected cell line were upregulated 15-fold by chronic nicotine exposure, but the ion flux activity increased only 45%, thus the amount of acetylcholine-induced ion flux per acetylcholine receptor was decreased (Gopalakrishnan et al., 1996). α3-Containing acetylcholine receptors are also upregulated, although much higher concentrations of nicotine are required. A mixture of α3-containing acetylcholine receptors expressed by the human neuroblastoma cell line SH-SY5Y increased by 600% in response to exposure to high concentrations of nicotine (Peng et al.,

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1997), and functional human α3β2 and α3β2α5 acetylcholine receptors in permanently transfected cell lines were increased up to 24-fold by chronic exposure to high concentrations of nicotine (Wang et al., 1998).

Chronic nicotine exposure causes inactivation of some acetylcholine receptor subtypes (Collins and Marks, 1996; Dani and Heinemann, 1996; Gopalakrishnan et al., 1996; Hsu et al., 1996; Fenster et al., 1997,1999; Olale et al., 1997; Ke et al., 1998). Using *X. laevis* oocytes as an expression system, we (Olale et al., 1997) have previously shown that 0.2 μM nicotine irreversibly inactivates most human α4β2 and α7 acetylcholine receptors but inhibits α3β2 acetylcholine receptors much less and more reversibly. This means that although α3 acetylcholine receptors are present in lower amounts in the brain than are α4 and α7 acetylcholine receptors, after chronic exposure to nicotine, α3 acetylcholine receptors may be able to play a greater role in acute responses to endogenous acetylcholine or subsequent doses of nicotine. α3 acetylcholine receptors also play a major role in synaptic transmission in peripheral autonomic ganglia. Tolerance for nicotine exhibited by tobacco users, as well as the behavioral effects of nicotine, may well reflect the sustained inhibition of α4 and α7 acetylcholine receptors in combination with the residual susceptibility of α3-containing acetylcholine receptors and related acetylcholine receptors. This hypothesis does not depend solely on studies of expressed cloned acetylcholine receptor subtypes. Rat striatal synaptosomes also show long-lasting inactivation of nicotinic acetylcholine receptor function after chronic treatment with micromolar concentrations of nicotine (Collins and Marks, 1996; Rowell and Duggan, 1998). Rowell and Duggan (1998), for instance, reported reversible desensitization after 10 min exposures to 0.3 μM nicotine but complete recovery within 1 h. However, 12 s of 30 μM nicotine resulted in 30% of inactivation not reversed after 1 h. They concluded that 50% of nicotine-induced dopamine release was due to α3β2 acetylcholine receptors that were not inhibited significantly, while the other half was due to α4β2 acetylcholine receptors that were mostly blocked.

It is not clear what molecular mechanisms are involved in the functional inactivation or upregulation associated with chronic exposure of acetylcholine receptors to nicotine. It is also unclear whether the two phenomena are the result of a single process. Conceivably, chronic exposure of acetylcholine receptors to nicotine may lead to a conformational change, altering ligand binding sites on the extracellular surface or affecting the transmembrane linkage to the channel gate near the cytoplasmic surface or other parts of the cytoplasmic surface. The effects of chronic nicotine exposure may instead or in addition be a result of covalent modification of the large cytoplasmic domain by mechanisms like phosphorylation, as has been proposed by Hsu et al. (1997) and Eilers et al. (1997). Acetylcholine receptor subunits contain potential phosphorylation sites in their large cytoplasmic domains (Miles and Huganir, 1988). Indeed, sequence analysis of α4 acetylcholine receptors subunits shows consensus sequences for phosphorylation by protein kinase A and tyrosine-specific protein kinase within the large cytoplasmic domain, and a protein kinase C phosphorylation site has been reported to regulate transitions between ''shallow'' and ''deep'' desensitized states (Fenster et al., 1999). α3 Acetylcholine receptor subunits have similar, but not identical, sites (Heinemann et al., 1991).

In this paper we investigate whether the extracellular domain, the transmembrane domains M1–M3 or the large cytoplasmic domain of α4 subunits play a direct role in the nicotine-induced functional inactivation of human α4β2 acetylcholine receptors. Nicotinic acetylcholine receptors are formed from five homologous subunits organized around a central cation channel (Karlin and Akabas, 1995; Wilson and Karlin, 1998; Lindstrom, 1996). Those formed from α7, α8 or α9 subunits can function as homomers. Acetylcholine receptors containing α2, α3, α4 or α6 subunits require β2 or β4 subunits and may also contain α5 or β3 subunits. Muscle acetylcholine receptors are thought to have their subunits organized in the order α1γα1δβ1 or α1εα1δβ1. Neuronal acetylcholine receptors are thought to similarly alternate α and β subunits so as to form two acetylcholine receptor binding sites at the interfaces between one side of each of two α subunits with the adjacent β subunit, e.g., α4β2α4β2β2 (Karlin, 1993; Lindstrom, in press). Acetylcholine receptor subunits exhibit signal sequences at the N-terminus that are cleaved during translation and that serve to target the large N-terminal domain to the extracellular surface. This extracellular domain precedes the putative transmembrane domains M1, M2 and M3. M1 is thought to provide part of the channel lining and may act as a linkage between agonist binding in the extracellular domain and the channel gate formed by the loop between M1 and M2. M2 provides most of the cation channel lining. In between M3 and M4 is the large cytoplasmic domain containing possible phosphorylation sites that may be involved in regulating acetylcholine receptor function, turnover or location. It is the most variable region of sequence among types of acetylcholine receptor subunits and among species for a given type of subunit. M4 comes immediately after the large cytoplasmic domain, and a small C-terminal extracellular domain is located at the end of each subunit.

α3 and α4 acetylcholine receptors exhibit different susceptibilities to nicotine-induced functional inactivation, and it is conceivable that these differences could be due to differences in their large cytoplasmic domains, such as differences in phosphorylation (Eilers et al., 1997; Hsu et al., 1997; Fenster et al., 1999). While most α4β2 acetylcholine receptors are irreversibly inactivated by a 24-h treatment with 0.2 μM nicotine, most α3 type acetylcholine receptors remain functional (Hsu et al., 1996; Olale et al., 1997; Wang et al., 1998). By constructing chimeras in which domains of the α3 and α4 subunits

have been switched, and then testing these chimeras for nicotine-induced functional inactivation, we can determine which domains of the α subunit determine susceptibility to the effects associated with chronic nicotine exposure. If only the large cytoplasmic domain determined this susceptibility, this would be consistent with a dominant role for phosphorylation of this part of the α subunit. However, if the large cytoplasmic domain were not sufficient to determine susceptibility to the effects of chronic nicotine exposure, then phosphorylation of the large cytoplasmic domain would not be the primary determinant and the relevant region would lie N-terminal to the cytoplasmic domain. In this case, conformational changes of the acetylcholine receptor associated with the extracellular domain or channel could provide plausible explanations of the mechanisms involved, as might covalent modification of the gate region between M1 and M2.

2. Materials and methods

2.1. Chimera construction

The cDNAs for human α4 subunit (Kuryatov et al., 1997), α3 subunit (Wang et al., 1996) and β2 subunit (Anand and Lindstrom, 1990) were cloned as previously described. The α4 (amino acid: 1 to Val 297)/α3 and α3(amino acid: 1 to Val 297)/α4 chimeras (hereafter referred to as $\alpha4_{1-297}/\alpha3_{298-446}$ and $\alpha3_{1-297}/\alpha4_{298-594}$, respectively) were constructed using an *Apa*LI restriction site common to the cDNAs of both subunits. The restriction site was picked so that the enzyme would cut right at Val 297, before the large cytoplasmic loop between M3 and M4 of each of the two subunits. The α4(amino acid: 1 to Arg 207)/α3 and α3(amino acid: 1 to Arg 207)/α4 chimeras (hereafter referred to as $\alpha4_{1-207}/\alpha3_{208-446}$ and $\alpha3_{1-207}/\alpha4_{208-594}$, respectively) were constructed using a *Bsp*EI site inserted without changing amino acid structure using PCR with GTACA*TCCGGA*GGACTGCC-CTTGTTC and T3 and CAGTC*TCCGGA*TGTACAGC-GAGTATG with T7 primers for α3 subunit, and CGT-CA*TCCGGA*GGCTGCCGCT with T3 and CAGCC*TC-CGGA*TGACGAAGG with T7 for α4. The chimeras were then cloned into a pSP64 (polyA) vector (Promega, Madison, WI) using *Hin*dIII and *Bam*HI cloning sites. An α4 mutant with its cytoplasmic domain deleted from His 298 up to Ala 562 (hereafter $\alpha4_{1-298}/(Gln)_{20}/\alpha4_{562-594}$) was constructed by cutting the α4 cDNA with *Apa*LI and *Nco*I enzymes to remove 261 amino acids, and replacing them with 20 Gln residues using the synthetic oligos TGCAC$(CAGCAA)_{10}$GC and CATGGC$(TTGCTG)_{10}$G. The chimeric cDNAs were checked for accuracy by restriction enzyme analysis and DNA sequencing prior to cRNA preparation. cRNAs were synthesized in vitro using the Megascript Kit (Ambion, Austin, TX). Schematic diagrams of the wild-type subunits and chimeras are shown in Fig. 1.

2.2. Oocyte isolation and cRNA injection

Oocytes were obtained from *X. laevis* (Xenopus I, Ann Arbor, MI). The oocytes were surgically removed and

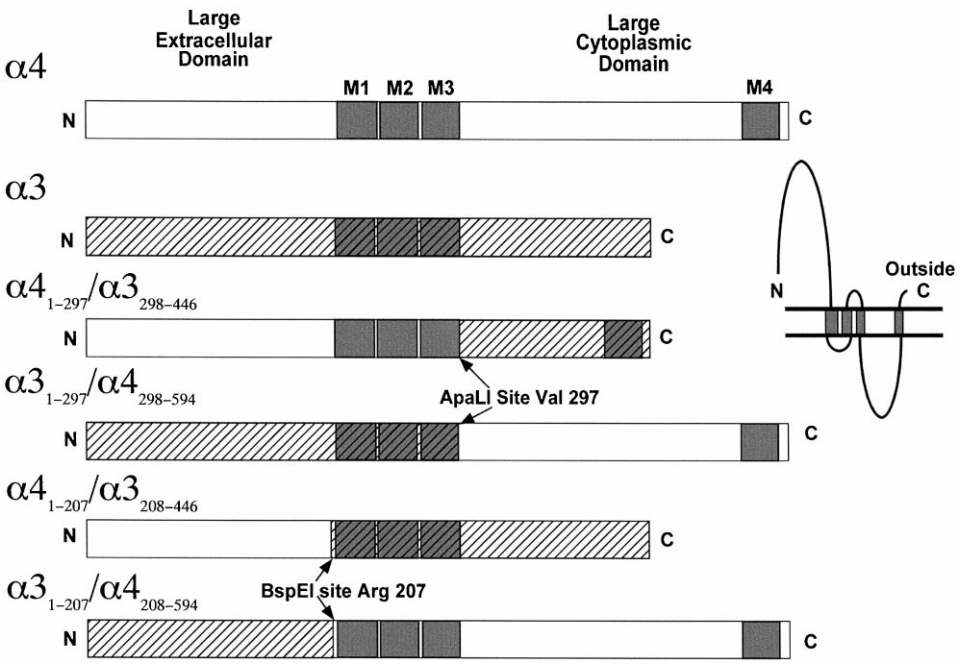

Fig. 1. Schematic diagrams of wild-type and chimeric α3 and α4 subunits. The $\alpha4_{1-297}/\alpha3_{298-446}$ and $\alpha3_{1-297}/\alpha4_{298-594}$ chimeras were constructed using an *Apa*LI restriction enzyme site at valine 297, common to each subunit cDNA. The $\alpha4_{1-207}/\alpha3_{208-446}$ and $\alpha3_{1-207}/\alpha4_{208-594}$ chimeras were constructed using a *Bsp*EI site inserted without changing the amino acid sequence. The insert depicts the transmembrane orientation of an acetylcholine receptor subunit polypeptide chain.

placed in diluted L-15 medium (50% Leibovitz's L-15 medium (Life Technologies, Grand Island, NY), 10 mM HEPES, pH 7.5 with NaOH) containing 50 U/ml penicillin, 50 μg/ml streptomycin and 50 μg/ml gentamicin. Oocytes were rinsed in calcium-free OR2 buffer (82.5 mM NaCl, 2 mM KCl, 1 mM $MgCl_2$, 5 mM HEPES, pH7.5 with NaOH) then defolliculated in this buffer containing 2 mg/ml collagenase A (Sigma, St. Louis, MO) for approximately 2 h.

Stage V–VI oocytes were selected and injected with 10 ng each of cRNAs for α and β2 subunits in a total volume of 20 nl, except for the low expressing chimera $\alpha3_{1-297}/\alpha4_{298-594}$, in which case 30 ng were used in a total volume of 40 nl. To study the upregulation effects from chronic exposure to nicotine, oocytes were injected with 2.5 ng each of cRNAs for the α and β2 subunits in a total volume of 10 nl. Following injections, oocytes were maintained semi-sterile at 18°C in diluted L-15 medium.

2.3. Oocyte surface acetylcholine receptor binding, solid-phase radioimmunoassay and nicotine-induced upregulation

Four days after injection, oocytes were rinsed in oocyte physiological saline (ND-96) (96 mM NaCl, 2 mM KCl, 1.8 mM $CaCl_2$ and 10 mM HEPES, pH 7.5). To assess surface expression of acetylcholine receptors, intact oocytes were incubated with gentle rotation in 1 ml ND-96 containing 10% horse serum (10% HS/ND-96) and 5 nM monoclonal antibody 295 to β2 subunits (Whiting and Lindstrom, 1988) for 2 h at 20°C. After washing 4 times with 10% HS/ND-96, oocytes were similarly incubated for 2 h with 5 nM ^{125}I-labeled goat-anti-rat antibodies in 10% HS/ND-96. After washing 4 times as above, oocytes were individually placed in a γ-counter (1277 Gammamaster, LKB Wallac) to measure bound ^{125}I-labeled goat-anti-rat antibodies. Nonspecific binding was determined by incubating noninjected oocytes under similar conditions.

To determine total acetylcholine receptor content in oocytes, solid-phase radioimmunoassay was performed using Immulon 4 microtiter wells (Dynatech, Chantilly, VA) coated with monoclonal antibody 295 as previously described (Anand et al., 1993). Oocytes were homogenized by repetitive pipetting in buffer containing 50 mM Na_2HPO_4–NaH_2PO_4, pH 7.5, 50 mM NaCl, 5 mM EDTA, 5 mM EGTA, 5 mM benzamidine, 15 mM iodoacetamide and 2 mM phenylmethylsulfonyl fluoride. Membrane fractions were collected by centrifugation for 20 min at 15,000 $\times g$. Acetylcholine receptors were solubilized by incubating the membrane fractions in the same buffer containing 2% Triton X-100 at 20°C for 1 h. Particulate material was removed by centrifugation for 20 min at 15,000 $\times g$. Triton-solubilized acetylcholine receptors were then added to monoclonal antibody 295-coated microtiter wells in 100 μl aliquots for overnight binding at 4°C. After binding, the wells were washed three times with cold PBS/0.05% Tween-20 buffer then incubated with 100 μl 5 nM ^{3}H-epibatidine (DuPont NEN, Boston, MA) for 2 h at 20°C. The wells were washed three times with PBS/0.05% Tween-20 buffer then eluted with buffer containing 3% SDS and 0.1 M dithiothreitol. Finally, the amount of radioactivity bound was determined by liquid scintillation counting using Optiphase ''Hi Safe'' 3 (Wallac, Finland). Non-specific binding was determined by radioimmunoassays with extracts from noninjected oocytes.

To study the effects of chronic nicotine treatment on the upregulation of acetylcholine receptors, on the third day after injection, oocytes were transferred to diluted L-15 medium containing 5 or 100 μM nicotine for 2 days. Non-treated oocytes were used as control. Oocytes were assayed for total acetylcholine receptor content as described above.

2.4. Nicotine treatment and electrophysiological recordings

Currents were measured using a standard two-microelectrode voltage clamp amplifier (Oocyte Clamp OC-725; Warner Instrument, Hamden, CT) as previously described (Gerzanich et al., 1995). All recordings were digitized using MacLab software and hardware (AD Instruments, Castle Hill, Australia) and stored on an Apple Macintosh IIcx computer. Data were analyzed using KALEIDAGRAPH (Synergy Software, Reading, PA) and fitted using a modified Hill equation.

The recording chamber was continually perfused at a flow rate of 10 ml/min with ND-96 containing 0.5 μM atropine. Application of the agonists was performed using a set of eight glass tubes as previously described (Gerzanich et al., 1995). For α4β2, $\alpha4_{1-297}/\alpha3_{298-446}\beta2$ and $\alpha4_{1-207}/\alpha3_{208-446}\beta2$, 30 μM acetylcholine was used to evoke responses. For combinations containing the α3 extracellular domain, 300-μM acetylcholine was used because of the lower acetylcholine affinity of α3 acetylcholine receptors. Acetylcholine was used rather than nicotine to mimic physiological conditions. The concentrations of acetylcholine were picked so that near maximum responses could be obtained repeatedly with 3–6 min washes between applications.

For nicotine incubations of less than 30 min, the oocytes remained in the recording chamber following the initial acetylcholine response. Then they were perfused with the saline solution containing the appropriate concentration of nicotine. For incubations longer than 30 min, the oocytes were removed from the chamber and incubated in a separate well of a 24-well tissue culture plate with diluted L-15 medium containing the appropriate concentration of nicotine. At various times, the oocytes were removed from the wells and returned to the recording chamber for measurement of acetylcholine responses.

3. Results

3.1. Chimeric acetylcholine receptor subunits efficiently form receptors when expressed with β2 subunits

Fig. 1 shows schematic diagrams of mature α4 and α3 AChR receptors subunit peptides and the chimeras formed by switching domains between α3 and α 4 subunits. The chimera termed $\alpha 3_{1-297}/\alpha 4_{298-594}$ contains α3 sequence from the N-terminus through the extracellular domain containing the acetylcholine binding site and through transmembrane domains M1–M3 containing the channel and its gate up to the start of the large cytoplasmic domain, followed by the remainder of the α4 sequence. Similarly, the chimera termed $\alpha 4_{1-297}/\alpha 3_{298-446}$ contains the N-terminal region of α4 and thereafter contains the α3 sequence. The chimera termed $\alpha 3_{1-207}/\alpha 4_{208-594}$ con-

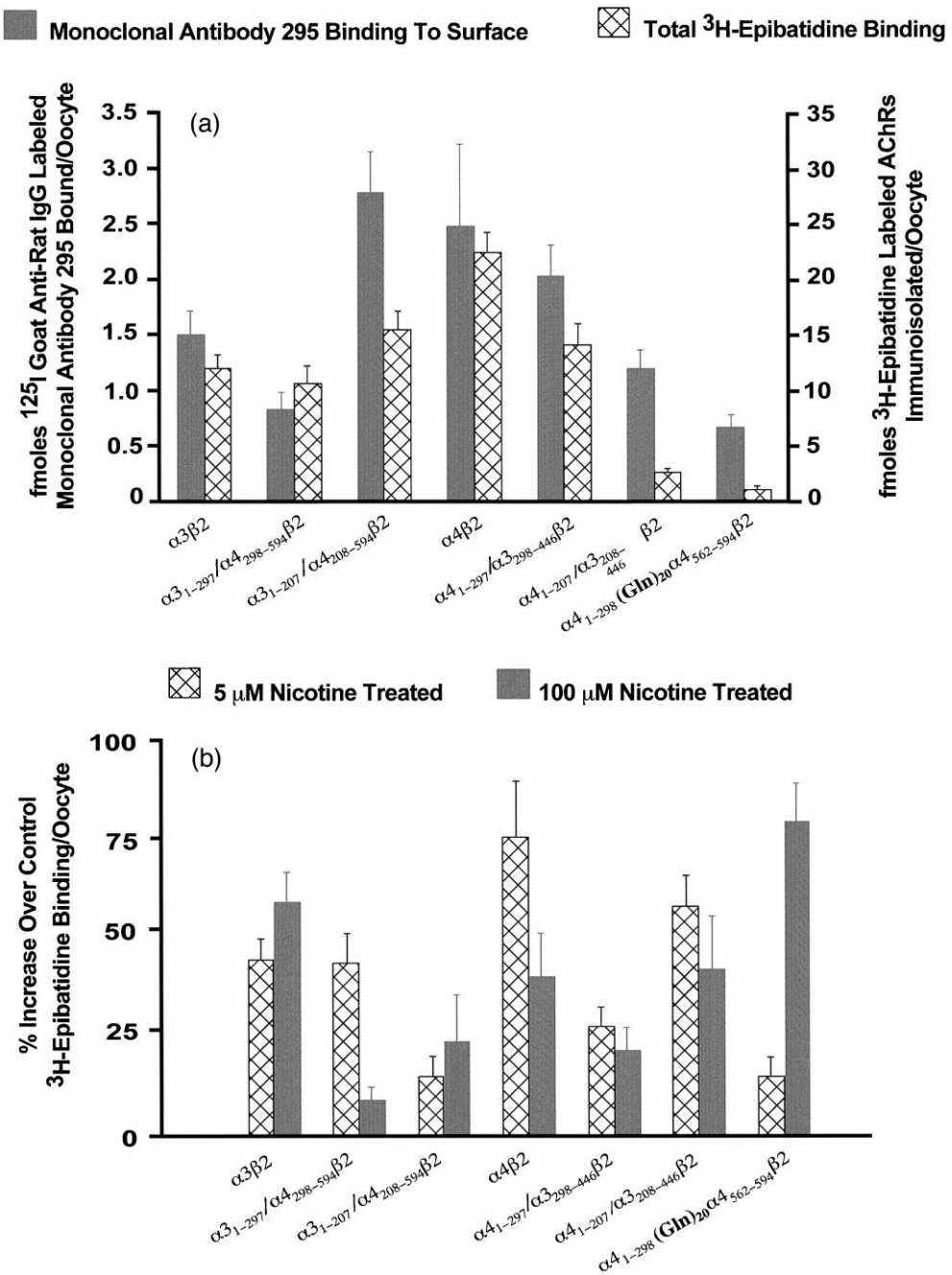

Fig. 2. Expression of acetylcholine receptors in *X. laevis* oocytes. (a) Most chimeric receptors except $\alpha 4_{1-207}/\alpha 3_{208-446}$ and $\alpha 4_{1-298}/(Gln)_{20}/\alpha 4_{562-594}$ were expressed at levels broadly similar to wild-type α3β2 and α4β2 receptors. ^{3}H-Epibatidine-labeled receptors were immunoisolated from detergent extracts of oocytes using wells coated with monoclonal antibody 295 (which is specific for β2 subunits). Data are an average of 15 assays consisting of triplicate extracts of 6–8 oocytes per subunit combination, except for $\alpha 4_{1-298}(Gln)_{20}\alpha 4_{562-594}$, which is an average of three assays of 6–8 oocytes each. (b) Upregulation of receptors after nicotine exposure for 2 days was observed for all receptors, including a mutant lacking the cytoplasmic domain of α4.

tains α3 sequence from the N-terminus only through the extracellular domain, followed by the remainder of the α4 sequence. Similarly, the chimera termed $\alpha4_{1-207}/\alpha3_{208-446}$ contains only the extracellular domain of α4 and thereafter contains the α3 sequence.

Most chimeric acetylcholine receptors were expressed in *X. laevis* oocytes at levels roughly similar to those for wild-type α4β2 and α3β2 acetylcholine receptors (Fig. 2a). This was shown both by using immunoprecipitation of detergent solubilized acetylcholine receptors labeled with ^{3}H-epibatidine to measure total acetylcholine receptors and by immunolabeling of intact oocytes followed by ^{125}I anti-IgG to assay receptors on the cell surfaces. Both assays used monoclonal antibody 295 that binds to the extracellular surface of β2 subunits (Whiting and Lindstrom, 1988). $\alpha4_{1-207}/\alpha3_{208-446}\beta2$ receptors were expressed at a lower level on the oocyte surface, which can explain the smaller currents and more variable inactivation observed with this chimera. The $\alpha4_{1-298}/(Gln)_{20}/\alpha4_{562-594}$ mutant, which lacks the large cytoplasmic domain, when expressed along with β2 subunits did not form functional acetylcholine receptors, although it did express a substantial amount of apparently properly assembled epibatidine binding sites and some receptors on the oocyte surface. Exposure to nicotine for 2 days caused an increase in the amount of native α3 and α4 acetylcholine receptors, all of the chimeras tested and even the α4 mutant lacking the large cytoplasmic domain (Fig. 2b).

3.2. Chimeric acetylcholine receptors retain the pharmacological properties of the wild-type receptor providing the extracellular and channel domains

As shown in Fig. 3, human α4β2 acetylcholine receptors are potently activated by both acetylcholine (EC_{50} = 1.9 ± 0.31 μM; nH = 0.9) and nicotine (EC_{50} = 0.3 ± 0.04 μM; nH = 1.2). Similarly, $\alpha4_{1-297}/\alpha3_{298-446}\beta2$ acetylcholine receptors had an EC_{50} of 1.3 ± 0.23 μM (nH = 0.83) for acetylcholine and 0.37 ± 0.07 μM (nH = 1.1) for nicotine, and $\alpha4_{1-207}/\alpha3_{208-446}\beta2$ acetylcholine receptors had EC_{50} values of 2.01 ± 0.22 μM (nH = 1.2) for acetylcholine and 0.71 ± 0.14 μM (nH = 1.0) for nicotine. In contrast, α3β2 acetylcholine receptors are less sensitive than α4β2 acetylcholine receptors to both acetylcholine and nicotine, with EC_{50} values of 76 ± 14 μM (nH = 1.0) for acetylcholine and 91 ± 17 μM (nH = 1.2) for nicotine. Similarly, $\alpha3_{1-297}/\alpha4_{298-594}\beta2$ acetylcholine receptors had EC_{50} values of 85 ± 13 μM (nH = 0.93) for acetylcholine and 32 ± 6.9 μM (nH = 1.0) for nicotine, and $\alpha3_{1-207}/\alpha4_{208-594}\beta2$ acetylcholine receptors had EC_{50} values of 93 ± 9.3 μM (nH = 1.0) for acetylcholine and 69 ± 22 μM (nH = 0.90) for nicotine.

Nicotine is a partial agonist on both native α3β2 and $\alpha3_{1-297}/\alpha4_{298-594}\beta2$ acetylcholine receptors, but is a full agonist on $\alpha3_{1-207}/\alpha4_{208-594}\beta2$ acetylcholine receptors. This suggests that a transmembrane region such as the channel of α3 determines the efficacy of nicotine in this

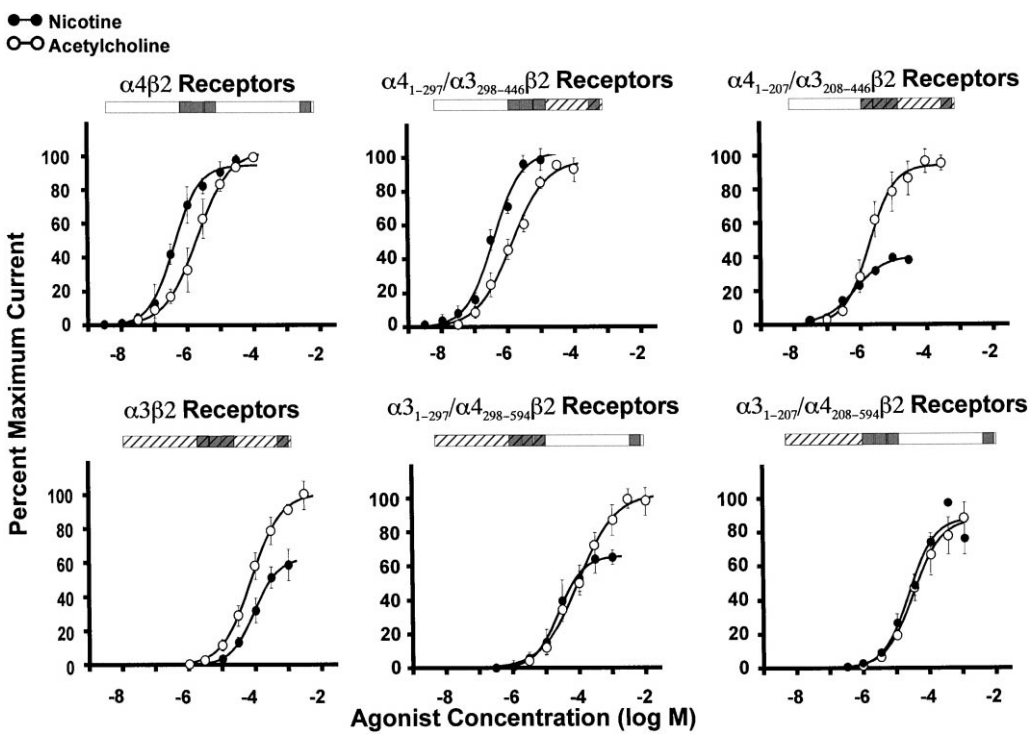

Fig. 3. Concentration dependence of acute activation of chimeric acetylcholine receptors by nicotine and acetylcholine. Oocytes were exposed to consecutive, increasing concentrations of ligand for 4 s at intervals of 4 min while continuously perfusing the recording chamber with saline. Concentration/response curves were fitted using a modified Hill equation. Each point represents responses from 4–6 oocytes clamped at −50 mV. Peak current response for each concentration was determined.

case. Consistent with this idea, nicotine is also a partial agonist on $\alpha4_{1-207}/\alpha3_{208-446}\beta2$ acetylcholine receptors (0.38 ± 0.01). The β subunit also contributes to efficacy, as shown by the observation that nicotine is a full agonist on $\alpha4_{1-207}/\alpha3_{208-446}\beta4$ acetylcholine receptors.

3.3. Chimeric $\alpha4_{1-297}/\alpha3_{298-446}\beta2$ and $\alpha4_{1-207}/\alpha3_{208-446}\beta2$ acetylcholine receptors, like wild-type $\alpha4\beta2$ acetylcholine receptors, are inactivated by chronic exposure to nicotine, but chimeric $\alpha3_{1-297}/\alpha4_{298-594}\beta2$ and $\alpha3_{1-207}/\alpha4_{208-594}\beta2$ acetylcholine receptors, like wild-type $\alpha3\beta2$ acetylcholine receptors, are resistant to inactivation

To determine the effects of long term exposure to nicotine on chimeric acetylcholine receptors, oocytes expressing $\alpha4_{1-297}/\alpha3_{298-446}\beta2$ acetylcholine receptors and $\alpha4\beta2$ acetylcholine receptors were incubated for 142 h in varying concentrations of nicotine then tested with 100 μM acetylcholine (Fig. 4). $\alpha4_{1-297}/\alpha3_{298-446}\beta2$ acetylcholine receptors were inactivated by chronic exposure to submicromolar concentrations of nicotine, just as we had previously observed with $\alpha4\beta2$ acetylcholine receptors (Olale et al., 1997) and confirmed here in parallel control experiments. The IC_{50} value for nicotine-induced loss of response was 48 ± 11 nM for $\alpha4_{1-297}/\alpha3_{298-446}\beta2$ acetylcholine receptors and 48 ± 8 nM for $\alpha4\beta2$ acetylcholine receptors. By contrast, both $\alpha3_{1-297}/\alpha4_{298-594}\beta2$ acetylcholine receptors ($IC_{50} = 670 \pm 11$ nM) and wild-type $\alpha3\beta2$ acetylcholine receptors ($IC_{50} = 450 \pm 100$ nM) were resistant to inactivation by chronic exposure to nicotine. Note that nicotine concentrations lower than neces-

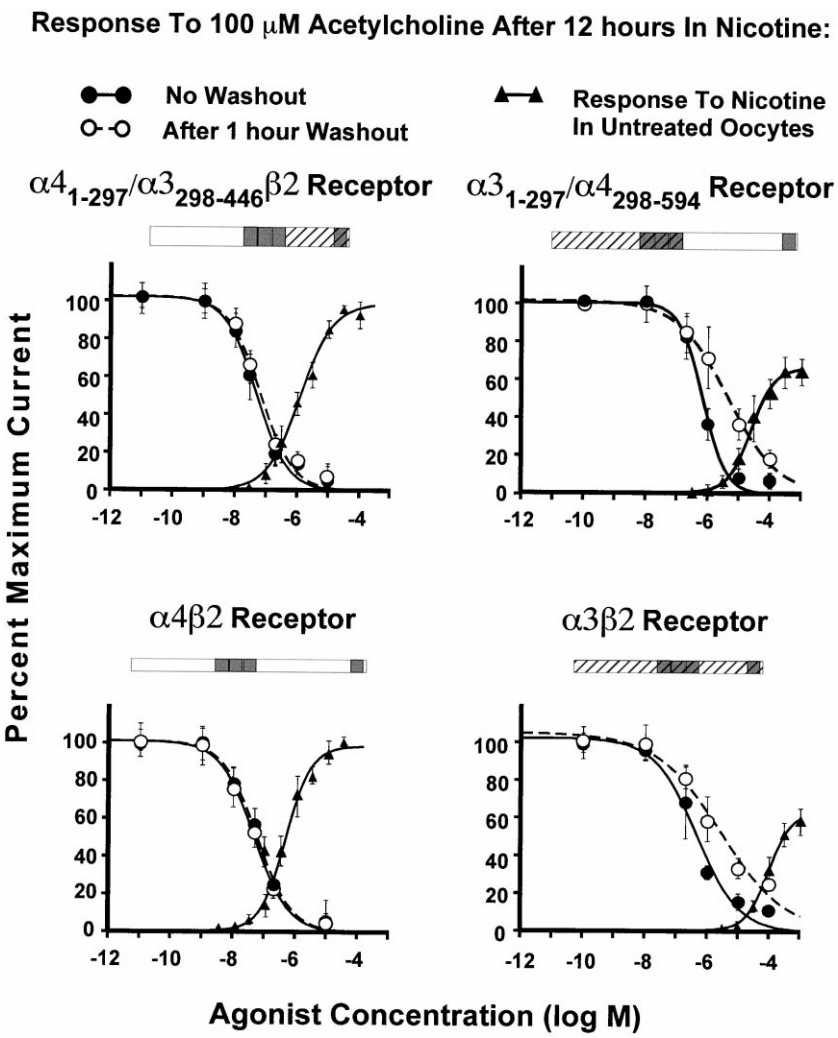

Fig. 4. Effects of chronic nicotine exposure on the function of $\alpha4_{1-297}/\alpha3_{298-446}\beta2$ and $\alpha3_{1-297}/\alpha4_{298-594}\beta2$ acetylcholine receptors. Acute activation curves are the same as in Fig. 3. For nicotine inhibition, control responses were elicited 3 days after oocyte injection. Oocytes were then incubated for 3 h at 18°C in varying concentrations of nicotine and then responses were evoked using 100-μM acetylcholine. Two normalized response inhibition curves are shown. One was obtained in the presence of the relevant nicotine concentration (no washout) and the other was obtained after a 1-h washout in saline. Responses from each oocyte after the nicotine incubation and the 1-h wash were compared to responses from the same oocyte, and the results from 3–6 oocytes were then averaged to obtain each point in the curve. Current responses were normalized to the maximum response on that day for oocytes not incubated in nicotine. Data obtained for $\alpha4\beta2$ and $\alpha3\beta2$ acetylcholine receptors are included for comparison.

sary for detectable acute activation are, when applied for 12 h, sufficient to inhibit the response to acetylcholine. Note also that, at any concentration of nicotine tested after 12 h, the inhibition of $\alpha4\beta2$ and $\alpha4_{1-297}/\alpha3_{298-446}\beta2$ acetylcholine receptors is not reduced by washing. However, both $\alpha3\beta2$ and $\alpha3_{1-297}/\alpha4_{298-594}\beta2$ acetylcholine receptors recover substantial activity after washing. We define reversible desensitization as inhibition that can be reversed by 1 h of washing and permanent inactivation as inhibition that cannot be reversed by this washing.

3.4. Chimeric $\alpha4_{1-297}/\alpha3_{298-446}\beta2$ acetylcholine receptors, like wild-type $\alpha4\beta2$ acetylcholine receptors, show faster and more extensive permanent inhibition after prolonged nicotine exposure, whereas chimeric $\alpha3_{1-297}/\alpha4_{298-594}\beta2$ and $\alpha3_{1-207}/\alpha4_{208-594}\beta2$, like wild-type $\alpha3\beta2$ acetylcholine receptors, show much less inactivation

Chimeric acetylcholine receptors expressing just the extracellular domain of $\alpha4$ or both the extracellular and trans-membrane channel domains of $\alpha4$ show nicotine-induced inactivation, though not as much as do wild-type $\alpha4\beta2$ acetylcholine receptors (Fig. 5). Correspondingly, chimeric acetylcholine receptors with only the cytoplasmic domain of $\alpha4$ or both the cytoplasmic and transmembrane channel domains of $\alpha4$ showed less nicotine-induced inactivation than wild-type $\alpha4\beta2$, but more than wild-type $\alpha3\beta2$ acetylcholine receptors. The time courses of nicotine-induced inhibition and recovery are shown in Fig. 5. Initial control currents were evoked 3 days after injection of cRNA. At this point the oocytes' ability to synthesize new acetylcholine receptors was expected to be substantially reduced due to cRNA decay. Acetylcholine receptors were incubated in 0.2 μM nicotine for different lengths of time. The concentration chosen for nicotine of 0.2 μM is the concentration of nicotine reported to be in the serum of smokers (Benowitz et al., 1990). At this concentration, as indicated by the dose/response curves in Fig. 3, nicotine would be expected to have more effect on the high affinity acetylcholine receptor binding sites in the extracellular domain of $\alpha4$ subunits than on the lower affinity sites of $\alpha3$ acetylcholine receptors. Response to 30 μm acetylcholine for $\alpha4$ acetylcholine receptors and to 300 μm for $\alpha3$ acetylcholine receptors was determined initially without nicotine and then sequentially in 0.2 μm nicotine after 1, 5, 30 and 180 min incubation, then again after 1 h wash in saline to permit recovery from reversible desensitization. Both wild-type $\alpha4\beta2$ acetylcholine receptors and $\alpha4_{1-297}/\alpha3_{298-446}\beta2$ acetylcholine receptors were inhibited by more than 70% after 3 h of incubation in nicotine. In contrast, there was practically no inhibition in $\alpha3\beta2$ acetylcholine receptors, and after 3 h incubation in nicotine

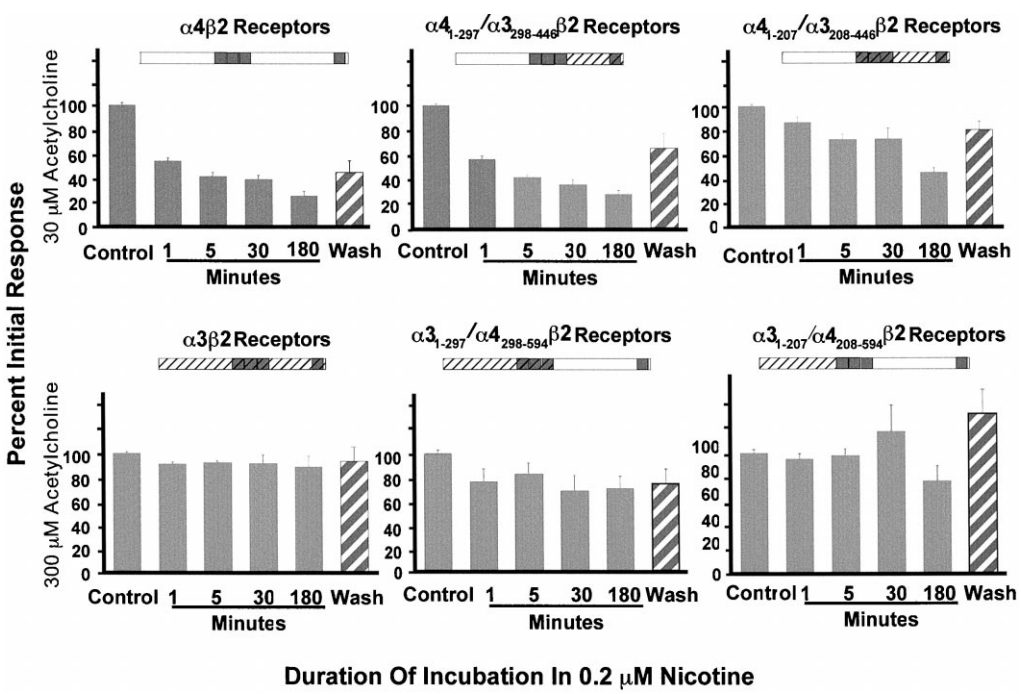

Fig. 5. Time-course of nicotine-induced inactivation of chimeric acetylcholine receptors. Initial maximum currents were determined 3 days after cRNA injection using 30-μM acetylcholine for receptors containing $\alpha4$ extracellular domains and 300 μM for receptors containing $\alpha3$ extracellular domains. Oocytes were then incubated in 0.2 μM nicotine for various times. Test responses for each individual oocyte were measured immediately after incubation in nicotine then compared to responses for the same oocyte prior to nicotine incubation. After the last incubation for 3 h in nicotine oocytes were washed for 1 h in diluted L-15 medium and the responses were measured again. Responses were normalized to responses for the same oocyte prior to nicotine. Each bar represents the mean of responses obtained from 5–7 oocytes.

$\alpha 3_{1-297}/\alpha 4_{298-594}\beta 2$ acetylcholine receptors were only inhibited by 25%.

4. Discussion

In this study, we show that both the acute and chronic electrophysiological responses to nicotine of $\alpha 3$ and $\alpha 4$ subunits are primarily determined by sequences N-terminal to the beginning of the large cytoplasmic domain. This shows that phosphorylation of sites C-terminal to the beginning of the large cytoplasmic domain cannot be solely responsible for the greater susceptibility of $\alpha 4\beta 2$ acetylcholine receptors than $\alpha 3\beta 2$ acetylcholine receptors to inactivation by chronic exposure to nicotine, although they could modulate this effect. Possible mechanisms by which nicotine might produce long-lasting inactivation include causing the conformational or covalent modification of the acetylcholine binding site, cation channel or gate, all of which are located in the region N-terminal to the beginning of the large cytoplasmic domain.

Acute responses of acetylcholine receptors to agonists, including activation and desensitization, depend on both α and β subunits (Papke, 1993). The two acetylcholine binding sites, for instance, are formed in the extracellular domain at interfaces between the N-terminal halves of two pairs of α and β subunits; the channel, on the other hand, is formed by membrane-spanning segments of all the subunits (Karlin and Akabas, 1995; Wilson and Karlin, 1998). Acute sensitivity to agonist binding is correspondingly determined by regions in the extracellular domain of the acetylcholine receptors (Bertrand and Changeux, 1995; Corringer et al., 1998). It is therefore not surprising that $\alpha 4_{1-297}/\alpha 3_{298-446}\beta 2$ and $\alpha 4_{1-207}/\alpha 3_{208-446}\beta 2$ chimeric acetylcholine receptors have EC_{50} values for activation by nicotine and acetylcholine that are close to those of $\alpha 4\beta 2$ acetylcholine receptors, while chimeric $\alpha 3_{1-297}/\alpha 4_{298-594}\beta 2$ and $\alpha 3_{1-207}/\alpha 4_{208-594}\beta 2$ acetylcholine receptors have EC_{50} values close to those of $\alpha 3\beta 2$ acetylcholine receptors, since in the construction of all chimeras acetylcholine receptor binding sites were left intact. Partial efficacy for nicotine correlates with the presence of the M1–M3 transmembrane domains of $\alpha 3$ subunits in combination with $\beta 2$ subunits. This is consistent with the idea that properties of the cation channel lined largely by the M2 domains of each subunit could determine the efficacy of nicotine, perhaps by providing low affinity binding sites at $\alpha 3/\beta 2$ interfaces through which nicotine could block the channel. Since nicotine is a full agonist on $\alpha 4_{1-207}/\alpha 3_{208-446}\beta 4$ acetylcholine receptors, it is likely that such binding sites would involve one or more of the only three amino acid residues at which $\beta 2$ and $\beta 4$ subunits differ in the M1–M3 region.

Chronic responses of acetylcholine receptors to agonists, including inactivation and upregulation, also depend on both α and β subunits (Fenster et al., 1997; Olale et al., 1997; Picciotto et al., 1998). $\alpha 3\beta 2$ acetylcholine receptors are more inhibited by prolonged exposure to 0.2 μM nicotine than are $\alpha 3\beta 4$ acetylcholine receptors (Fenster et al., 1997; Olale et al., 1997). In both a neuroblastoma line and in transfected cell lines, $\alpha 3\beta 2$ acetylcholine receptors exhibit more nicotine-induced upregulation than do $\alpha 3\beta 4$ acetylcholine receptors (Wang et al., 1998). It has also recently been reported that the $\beta 2$ subunit contributes to the reinforcing properties of nicotine in the mesolimbic dopamine system in mice (Picciotto et al., 1998). In our present work, we have chosen to use $\beta 2$ in all cases.

Previously, we and others have shown that chronic nicotine exposure differentially affects $\alpha 3$, $\alpha 4$ and $\alpha 7$ neuronal nicotinic acetylcholine receptors (Fenster et al., 1997; Hsu et al., 1996; Olale et al., 1997). Human $\alpha 4\beta 2$ and $\alpha 7$ acetylcholine receptors chronically exposed to submicromolar concentrations of nicotine were functionally inactivated. However, under the same conditions, $\alpha 3\beta 2\beta 4\alpha 5$ acetylcholine receptors were less sensitive to functional inactivation, and indeed recovered from a 20% reduction in function within an hour (Olale et al., 1997). Hsu et al. (1996) similarly showed that rat $\alpha 3\beta 2$ acetylcholine receptors were less sensitive to functional inactivation by chronic exposure to submicromolar concentrations of nicotine than were rat $\alpha 4\beta 2$ acetylcholine receptors. It has been suggested that functional inactivation caused by nicotine may be mediated through a protein kinase dependent mechanism. Hsu et al. (1997) showed in *Xenopus* oocytes that nicotine enhances the cyclic AMP-dependent protein kinase-mediated phosphorylation of $\alpha 4$ subunits. Phosphorylation of the $\alpha 4$ subunits of $\alpha 4\beta 2$ acetylcholine receptors increased within the first 5 min of incubation with nicotine and persisted for 24 h. The time of onset for phosphorylation was similar to that of inactivation, suggesting that the two processes may be related, but not necessarily proving that phosphorylation is the primary cause of functional inactivation.

Eilers et al. (1997) suggested that a protein kinase C-dependent mechanism mediates nicotine-induced inactivation of $\alpha 4\beta 2$ acetylcholine receptors. They reported that in permanently transfected HEK cells functional inactivation could be caused by inhibiting protein kinase C activity. They also argue that inhibitors of protein kinase A, the protein kinase implicated in Hsu et al. (1997) study, had no effect on acetylcholine receptor function.

Thus, Hsu et al. (1997) and Eilers et al. (1997) suggest opposing mechanisms by which phosphorylation, presumably of sites in the large cytoplasmic domain of the $\alpha 4$ subunit, might mediate nicotine-induced inactivation.

Fenster et al. (1999) reported that rat $\alpha 4\beta 2$ acetylcholine receptors expressed in *Xenopus* oocytes contain a protein kinase C consensus phosphorylation site in the $\alpha 4$ large cytoplasmic domain which, when inactivated by converting serine 336 to alanine, slowed recovery from desensitization by 30 min exposures to 0.3 μM nicotine. Conversely, inhibition of phosphatase by cyclosporin A

speeded recovery from desensitization. Thus, phosphorylation of this site in the α4 large cytoplasmic domain may speed recovery from desensitization.

Nonetheless, our data show that acetylcholine receptors in which the entire cytoplasmic domain of α4 subunits has been replaced with that of α3 subunits are functionally inactivated by nicotine as is characteristic of wild-type α4β2 acetylcholine receptors ($\alpha4_{1-297}/\alpha3_{298-446}\beta2$ acetylcholine receptors in Fig. 5). Correspondingly, acetylcholine receptors in which the entire cytoplasmic domain of α3 subunits has been replaced with that of α4 subunits exhibit little nicotine-induced functional inactivation, most closely resembling wild-type α3β2 acetylcholine receptors ($\alpha3_{1-297}/\alpha4_{298-594}\beta2$ acetylcholine receptors in Fig. 5). Thus, although phosphorylation at one or more of the several phosphorylation sites in the large cytoplasmic domain of α4 may modulate the rate of recovery from desensitization, much of the characteristic difference in nicotine-induced functional inactivation between α4 and α3 results from properties of the extracellular and transmembrane regions.

Our studies here and previously (Olale et al., 1997) indicate that chronic exposure to low concentrations of nicotine and other agonists can inactivate a large fraction of α4β2 and α7 acetylcholine receptors, but have a much smaller effect on α3β2 acetylcholine receptors. This suggests that normal acetylcholine mediated signaling through α4β2 and α7 acetylcholine receptors would be inhibited, whereas pathways using α3β2 acetylcholine receptors or other acetylcholine receptor subtypes which might remain similarly resistant to inactivation, would remain responsive both to normal acetylcholine-mediated signaling and to nicotine or other agonists. Inactivation of some acetylcholine receptor subtypes by chronic exposure to agonist provides a mechanism to explain tolerance, which is known to develop to some effects of nicotine. It seems likely that the addictive effects as well as some of the other effects of nicotine are mediated by agonist activity on acetylcholine receptor subtypes that are not extensively inactivated by chronic exposure to nicotine. It is also important to consider that in the presence of a concentration of nicotine or other agonists that would inactivate most α4β2 or α7 acetylcholine receptors, the fraction remaining functional would be exposed to agonist for times tens of millions-fold longer than the millisecond or so normally involved in synaptic transmission. Even a few percent of functional acetylcholine receptors exposed for a long time to an agonist concentration capable of producing only a few percent of a maximum acute response might nonetheless cause significant accumulation of intracellular calcium even though the net effect on the membrane potential would be small. This might trigger long term effects on function, growth and gene regulation of the cells expressing these acetylcholine receptors.

The contrast among the activating, desensitizing, inactivating and upregulating effects of agonists on acetylcholine receptors combined with the huge difference in time of exposure to synaptically released acetylcholine and agonist drugs make it very difficult to predict whether a nicotinic drug will have a net agonist or antagonist effect on various pathways in vivo. The effects will undoubtedly depend on which acetylcholine receptor subtypes mediate the many effects of nicotine. α4β2 and α7 acetylcholine receptors are the predominant acetylcholine receptors subtypes found in the mammalian brain. Many peripheral ganglionic neurons express both α7 and α3 acetylcholine receptors, and in at least one case either post-synaptic α3 acetylcholine receptors or peri-synaptic α7 acetylcholine receptors can mediate transmission (Zhang et al., 1996; Ullian et al., 1997). Minor subtypes containing the α6 subunit, which is very closely related in sequence to the α3 subunit (Le Novere et al., 1996), may mediate important processes and become specific drug targets. For example, α6 is found in the substantia nigra (which is damaged in Parkinson's disease) and in the ventral tegmental area (which is involved in addiction) (Goldner, 1997; Le Novere and Changeux, 1995). Thus, it will be important to also determine the effects of chronic exposure to nicotinic agonists on such minor acetylcholine receptor subtypes.

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