α5 Subunit Alters Desensitization, Pharmacology, Ca\textsuperscript{2+} Permeability and Ca\textsuperscript{2+} Modulation of Human Neuronal α3 Nicotinic Receptors\textsuperscript{1}

VOLODYMYR GERZANICH, FAN WANG, ALEXANDER KURYATOV and JON LINDSTROM

Department of Neuroscience, University of Pennsylvania Medical School, Philadelphia, Pennsylvania

Accepted for publication March 2, 1998 This paper is available online at http://www.jpet.org

ABSTRACT

Functional effects of human α5 nicotinic ACh receptor (AChR) subunits coassembled with α3 and β2 or with α3 and β4 subunits, were investigated in *Xenopus* oocytes. The presence of α5 subunits altered some properties of both α3 AChRs and differentially altered other properties of α3β2 AChRs vs. α3β4 AChRs. α5 subunits increased desensitization and Ca\textsuperscript{2+} permeability of all α3 AChRs. The Ca\textsuperscript{2+} permeabilities of both α3β2α5 and α3β4α5 AChRs were comparable to that of α7 AChRs. As we have shown previously, α5 subunits increased the ACh sensitivity of α3β2 AChRs 50-fold but had little effect on α3β4 AChRs, α5 caused only subtle changes in the activation potencies of α3 AChRs for nicotine, cytisine and 1,1-dimethyl-4-phenylpiperazinium (DMPP). However, α5 increased the efficacies of nicotine and DMPP on α3β2 AChRs but decreased them on α3β4 AChRs. Immunoprecipitation studies showed that α5 efficiently coassembled with α3 plus β2 and/or β4 subunits. As expected, human AChRs immunoprecipitated with SH-SYSY neuroblastoma cells showed that AChRs containing α5 and probably α5 subunits were present, but α4 AChRs were not. In brain, by contrast, α4β2 AChRs were shown to predominate over α3 AChRs. Some of the brain α4β2 AChRs were found to contain α5 subunits.

Neuronal nicotinic AChRs are thought to be formed by pentameric assemblies of certain combinations of α2, α3, α4, α5, α6, α7, α8, α9, β2, β3 and β4 subunits (Deneris et al., 1991; Role, 1992; Sargent, 1993; Le Novere and Changeux, 1995; Lindstrom et al., 1995; McGehee and Role, 1995; Lindstrom, 1996). The homologous subunits of an AChR are thought to be organized around a central cation channel like barrel staves so that parts of the M1 and M2 transmembrane domains of all subunits contribute to the lining of the channel. In the case of muscle-type AChRs, which are known to have their subunits organized around the channel in the order α1γβ1δ, there are two ACh binding sites at interfaces between α and γ or between α and δ subunits, but the β1 subunit is not thought to contribute contact amino acids to these binding sites (Karlin and Akabas, 1995). The stoichiometry of α4β2 AChRs expressed in oocytes is known to be (α4)\textsubscript{2} (β2)\textsubscript{1} (Anand et al., 1991; Cooper et al., 1991), and it is thought that these subunits are similarly organized around the channel in the order α4β2α4β2β2, which results in two ACh binding sites at interfaces between α4 and β2 subunits. α3 subunits can form functional AChRs in combination with β2 or β4 subunits, and it is presumed that these also probably have two ACh binding sites. α5 is known to be a subunit of AChRs containing α3, β4 and/or β2 subunits in chick ganglia (Conroy et al., 1992; Vernallis et al., 1993), in a human neuroblastoma (Wang et al., 1996), and associated with a small fraction of the α4β2 AChRs in chick brain (Conroy and Berg, 1995). The stoichiometry of α5 containing AChRs has not been directly determined. However, the observation that α5 does not form functional AChRs when expressed in *Xenopus* oocytes alone or in paired combination with α3, β2 or β4 (Wang et al., 1996) suggests that α5 subunits, like β1 subunits, cannot interface with the sides of these subunits that are involved in forming ACh binding sites (Karlin and Akabas, 1995). Thus it has been suggested that α5 may occupy a position homologous to that of β1 in muscle-type AChRs (Wang et al., 1996). For example, the order of subunits around the channel might be α3β2α3β2α5.

Our initial studies of human α5 subunits expressed in *Xenopus* oocytes showed that they assembled efficiently with human α3 and β2 or human α3 and β4 subunits to form AChRs that desensitized more rapidly and that, especially in the case of α3β2α5 AChRs, exhibited altered pharmacological properties (Wang et al., 1996). Here we extend these electrophysiological studies in *Xenopus* oocytes and conduct immunoprecipitation studies to investigate the fraction of various

**ABBREVIATIONS:** AChR, acetylcholine receptor; mAb, monoclonal antibody; DMPP, 1,1-dimethyl-4-phenylpiperazinium.
AChR subunits in extracts of rat and human brain that have α5 associated with them.

Materials and Methods

cDNAs. The cDNA sequences for human α3 (unpublished EMBL accession no. X53559) and β2 (Anand and Lindstrom, 1990) were subcloned in expression vectors pcDNAI (Invitrogen, San Diego, CA) and pSP64poly(A) (Promega, Madison, WI), respectively. The cDNA for human α5 was first described by Chini et al. (1992) and was kindly provided by Dr. Francesco Clementi (University of Milan). It was subcloned in the pSP64poly(A) vector. The cDNA for human β4 was cloned in this lab from a cDNA library from the neuroblastoma cell line SH-SY5Y (Gerzanich et al., 1997). It was then subcloned into the pcDNAI vector. α1 and δ cDNAs were described previously (Luther et al., 1989). Epitope tagged α5 cDNA was described previously (Wang et al., 1996). Human β, ε and γ cDNAs were kindly provided by Dr. Andrew Engel (Mayo Clinic).

Expression of human α3 AChRs in Xenopus oocytes. cDNAs for human AChR subunits α3, β2, β4 and α5 were synthesized in vitro using T7 (if the cDNA was in the pcDNAI vector) or SP6 (if the cDNA was in the pSP64poly(A) vector) RNA polymerase (mMESSAGEmACHINE, Ambion, Austin, TX). Oocytes were prepared for microinjection as described previously (Gerzanich et al., 1995) and injected with equal amounts (5–15 ng) of cRNA for each of the subunits. They were incubated for 3 to 4 days after injection in media containing 50% L15 (GIBCO BRL), 10 mM HEPES buffer, pH 7.5, 10 U/ml penicillin and 10 mg/ml streptomycin at 18°C.

Electrophysiologic procedures and drug application. Currents in oocytes were measured using a standard two-microelectrode voltage-clamp amplifier (Oocyte Clamp OC-725, Warner Instrument Corp., Hamden, CT). Electrodes were filled with 3 M KCl and had resistances of 0.5 to 1.0 MΩ for the voltage electrode and 0.4 to 0.6 MΩ for the current electrode. All records were digitized (MacLab/2e interface and Scope software (AD Instruments, Castle Hill, Australia), stored on a Macintosh IICx computer and analyzed using AXOGRAPH software (Axon Instruments, Foster City, CA). The recording chamber was continually perfused at a flow rate of 10 ml/min with saline solution containing 96 mM NaCl, 2 mM KCl, 1.8 mM CaCl₂, 1 mM MgCl₂, 5 mM HEPES, pH 7.6. Atropine (0.5–1 μM) was included in all solutions to block responses of endogenous muscarinic AChRs. Application of agonists was performed as described in detail previously (Gerzanich et al., 1995). In summary, all agonists were applied by means of a set of 2-mm glass tubes directed on the animal pole of the oocyte. Application was achieved by manual unclamping and clamping of a flexible tube connected to the syringe with the test solution. Typically delay between beginning of the application and first deflection of the induced current was about 0.25 sec. The Hill equation was fitted to the concentration-response dependence using a nonlinear least-squares error curve fit method (KaleidaGraph, Abelbeck Software): \[ I(x) = I_\text{max} \times \frac{[x]^{n} \times EC_{50}^{n}}{[x]^{n} + EC_{50}^{n}} \]
where \( I(x) \) is current measured at the agonist concentration \( x \), \( I_{\text{max}} \) is the maximal current response at the saturating agonist concentration, EC₅₀ is the agonist concentration required for the half-maximal response and \( n \) is the Hill coefficient.

For experiments measuring the effect of extracellular Ca²⁺ on the current amplitude and reversal potentials, intracellular electrodes were filled with 2.5 M potassium aspartate. In order to prevent activation of the endogenous Ca²⁺-dependent Cl⁻ channels, Cl⁻-free solutions were used for oocyte preincubation (6–12 hr) and for the perfusion during recordings (Francis and Papke, 1996). The "normal" Ca²⁺ solution included 90 mM Na₂SO₄, 2.5 mM KOH, 10 mM HEPES and 1.8 mM Ca(OH)₂. Additionally, 48 mM dextrose was supplemented in the normal solution in order to yield osmolarity equal to the "high" Ca²⁺ solution, which contained 18 mM Ca(OH)₂ and the same concentration of the other ions as the "normal" Ca²⁺ solution. Both solutions were buffered with methanesulfonic acid to pH 7.3. Reversal potentials of the currents were determined either by 6-sec agonist applications at different holding potentials or by 2-sec ramps of the holding potential from −50 to +50 mV during agonist application after the current reached a steady state. Both protocols gave similar estimates for the reversal potential. Control ramp currents obtained before agonist applications were subtracted from the ramp currents during AChR activation.

Purification and radioimmunoassay of AChRs from oocytes, SH-SYSY cells and human brain. Purification, immunodepletion and solid phase radioimmunoassay of AChRs from oocytes were performed as described previously (Wang et al., 1996). AChRs from the human neuroblastoma cell line SH-SYSY, neocortex from post-mortem human brain and whole rat brain tissue were isolated in accordance with the methods of Whiting and Lindstrom (1986) and Wang et al. (1996). For radioimmunoassay, 250-μl aliquots of tissue extract either were mixed directly with 50 μl of the mAb-Actigel and \(^{[3]H}\)-epibatidine (5.3 nM) or were preabsorbed with 50 μl of mAb-Actigel before mixing with \(^{[3]H}\)-epibatidine and a fresh aliquot of the mAb-Actigel. mAb-Actigel contained 5 mg/ml of mAb. After 8 to 12 hr of incubation at 4°C, the Actigel was rinsed three times with ice-cold PBS, 0.05% Tween buffer. The amount of bound AChRs was determined by labeling with 5 nM \(^{[3]H}\)-epibatidine, followed by liquid scintillation counting (Wang et al., 1996). Nonspecific binding of the AChRs to mAb-Actigel was determined by incubation of aliquots of tissue extracts with an irrelevant Ab or normal rat IgG-Actigel under the same conditions.

Results

α5 subunit enhances desensitization in recombinant human neuronal α3 AChRs. The time course of the currents induced by saturating concentrations of ACh in oocytes expressing AChRs after coinjection of α3β2α5 or α3β4α5 cRNA combinations are compared with those after α3β2 and α3β4 cRNA coinjections in figure 1. ACh-evoked currents reached a maximum and then decayed biphasically, showing both a transient and a plateau phase. Small “rebound” currents, commonly explained as channel block by agonist, were observed only for AChRs containing β4 subunits (fig. 1, bottom two traces). The onset of the current in the AChRs containing β2 subunits (fig. 1, top two traces) was significantly steeper (0.23 ± 0.1 and 0.17 ± 0.06 sec to peak for α3β2 and α3β2α5 combinations, respectively) compared to β4 subunit-containing AChRs (0.77 ± 0.34 and 0.43 ± 0.23 sec to peak for α3β4 and α3β4α5, respectively) (bottom two traces). Listed data represent the mean of 7 to 9 oocytes for each subunit combination ± S.D. Resolution of the current onset for β2-containing AChRs was limited by the perfusion time (see “Materials and Methods”).

Addition of α5 subunits to the α3β2 combination resulted in AChRs with notably faster desensitization. T₅₀ of the current decay upon exposure to a saturating concentration of ACh decreased from 1.1 to 0.64 sec (fig. 1, left plot on the top panel). In addition, the amount of desensitization (percent of current from the peak to plateau) increased from 46% to 68% (fig. 1, right plot on the top panel). A similar phenomenon was observed when α5 subunits were coexpressed together with α3 and β4 subunits. Both the rate of desensitization (T₅₀ of decay decreased from 1.8 to 0.7 sec) and amount of desensitization (increased from 21% to 41%) were enhanced in α3β4α5 compared with α3β4 AChRs (fig. 1, bottom panel).

α5 subunit alters pharmacology of recombinant human neuronal α3 AChRs. Pharmacological profiles of α3 AChRs were investigated using four nicotinic agonists: ACh, nicotine, cytisine and DMPP. Concentration-response curves...
for these agonists were built from data collected from oocytes expressing four different a3 neuronal AChR subtypes (fig. 2). Concentration-response curves for ACh and nicotine, which are shown for comparison with the effects of DMPP and cytisine, are from our previous study (Wang et al., 1996). All currents were normalized to the maximal currents induced by ACh for each AChR subtype. ACh was used for normalization of efficacy of the nicotinic agonists because it is the endogenous agonist. Values for the EC_{50}, Hill coefficients and the relative maximal responses are listed in table 1. Comparison of the families of the concentration/response curves built for a3b2, a3b2a5, a3b4 and a3b4a5 AChRs revealed striking differences in pharmacological properties among these AChRs.

Substitution of b2 subunits for b4 subunits in a3 AChRs resulted in decreases of potency for ACh, nicotine and DMPP (table 1). Furthermore, this resulted in increased efficacy of nicotine, changing it from a partial to a full agonist. Efficacy for cytisine also increased from 23% to 56% with no significant changes in apparent affinity. In addition, concentration-response curves for the agonists tested had higher Hill coefficients for a3b4 AChRs than for a3b2 AChRs.

Notable changes in pharmacological properties were observed when a5 subunits were added to a3b2 AChRs (fig. 3; table 1). Thus, as we have shown previously (Wang et al., 1996), a3b2a5 AChRs had almost 50 times higher sensitivity to ACh compared with a3b2 AChRs. Less significant increases of apparent affinity were observed for nicotine and DMPP. In contrast, efficacies of these agonists changed dramatically, nicotine switching from a partial (55%) to a full agonist (Wang et al., 1996), and DMPP increasing in efficacy from 107% to 187% compared with ACh. This, in essence, converted ACh and nicotine into partial agonists.

Addition of a5 subunits to a3b4 AChRs caused less significant changes in apparent affinities for the agonists tested (fig. 2; table 1). Only cytisine exhibited a moderate increase of apparent affinity for a3b4a5 AChRs compared with a3b4 AChRs, and there was basically no change in the rank order of potencies of agonists. In contrast to a3b2 AChRs, where addition of a5 subunits increased the efficacy of DMPP to greater than that of ACh, addition of a5 to a3b4 AChRs decreased the efficacy of DMPP from 100% to 13%. Overall, concentration-response curves for a3b4a5 AChRs had higher Hill coefficients than curves built for a3b2a5 AChRs (table 1).

a5 subunits enhance Ca^{++} permeability and Ca^{++} modulation of recombinant human neuronal a3 AChRs. Relative permeability of Ca^{++} through AChRs was evaluated by the shifts of reversal potential caused by changes in extracellular Ca^{++} concentration. More precise estimates of the permeability ratios were constrained by our inability to monitor intracellular cation concentrations while using the two-electrode voltage-clamp method. a7 AChRs were shown previously to have exceptionally high permeability for Ca^{++} ions, comparable to that of NMDA receptors (Bertrand et al., 1993; Seguela et al., 1993; Castro and Albuquerque 1995; Delbono et al., 1997). In contrast, muscle AChRs have rather low Ca^{++} permeability (Vernino et al., 1992; Dani and Mayer 1995; Francis and Papke 1996). These two AChRs were used to "calibrate" the range of the extracellular Ca^{++}-dependent shift of reversal potential (fig. 4) and, subsequently, to compare the relative Ca^{++} permeabilities of a3 AChR subtypes. Human a7 AChRs exhibited a 17.8 ± 0.9 mV (n = 12) positive shift of reversal potential as a result of a 10-fold increase of Ca^{++} concentration from 1.8 to 18 mV. Human muscle AChRs formed from a1, b1, c, d and e subunits exhibited a shift of only 0.8 ± 0.9 mV (n = 4). a3b2 and a3b4 AChRs had similar shifts of reversal potential upon increase of Ca^{++} concentration (5.8 ± 0.8 mV (n = 7) and 6.1 ± 1.2 mV (n = 6), respectively). This suggests similar contributions by both b2 and b4 subunits to the AChR channel lining. Incorporation of a5 subunits in both a3b2a5 and a3b4a5 AChRs dramatically increased the Ca^{++}-dependent...
shift of the reversal potential to 13.7 ± 1.4 mV (n = 11) and 11.7 ± 1.1 mV (n = 10), respectively. This indicates that the Ca\(^{2+}\) permeabilities of human α3β2α5 and α3β4α5 AChRs approach that of homomeric α7 AChRs.

Increase of the extracellular Ca\(^{2+}\) concentration also augmented the amplitude of currents mediated by α3 AChRs (fig. 4). Although for α3β4 AChRs this increase of amplitude could be attributed solely to the increase in the driving force due to the change of the reversal potential upon increase of the Ca\(^{2+}\) concentration, for α3β2 AChRs, the increase of amplitude in 18 mM Ca\(^{2+}\) was 3-fold larger. Addition of α5 subunits increased the α3β2 AChR-mediated current, whereas no increase was observed for α3β4 AChRs. Thus β2 and β4 subunits clearly contributed differently to extracellular Ca\(^{2+}\) modulation of α3 AChRs, and α5 further enhanced this modulation for α3β2α5 AChRs.

**Evidence that α5 subunits can assemble in AChRs with four different subunits.** Neurons frequently express α3, β2, β4 and α5 subunits (e.g., Conroy and Berg, 1995; Wang et al., 1996). Co-injection of equal amounts of all four subunit cRNAs—α3, β2, β4 and α5—resulted in AChRs that responded to ACh application in a distinct manner. The time

### Table 1

<table>
<thead>
<tr>
<th></th>
<th>ACh***</th>
<th>Nicotine***</th>
<th>Cytisine</th>
<th>DMPP</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>EC(_{50})*</td>
<td>nH</td>
<td>EC(_{50})*</td>
<td>nH</td>
</tr>
<tr>
<td>α3β2</td>
<td>26 ± 0.3</td>
<td>0.9</td>
<td>6.8 ± 0.5</td>
<td>1.0</td>
</tr>
<tr>
<td>α3β2α5</td>
<td>0.5 ± 0.6</td>
<td>1.4</td>
<td>1.9 ± 0.3</td>
<td>1.5</td>
</tr>
<tr>
<td>α3β4</td>
<td>163 ± 6</td>
<td>1.9</td>
<td>106 ± 4</td>
<td>2.0</td>
</tr>
<tr>
<td>α3β4α5</td>
<td>122 ± 20</td>
<td>1.7</td>
<td>105 ± 4.0</td>
<td>2.0</td>
</tr>
</tbody>
</table>

* Values from Wang et al. (1996) are shown for comparison.

**Maximum currents normalized to the maximum current induced by a saturating concentration of ACh.**
course of activation and desensitization of currents from α3β2β4α5 AChRs (fig. 5) resembled most closely the time course of α3β4α5 AChRs (fig. 1), though current rise and decay were both slower. Higher concentrations of ACh were required in order to saturate the response, and a small rebound current was observed upon removal of 3 mM ACh (fig. 5). The concentration-response curve yielded a satisfactory fit with a two-site Hill equation. The higher-affinity site (S1), with an EC50 of 24 mM, constituted ~35% of the maximal response. The lower-affinity affinity site (S2), with an EC50 of 345 mM, constituted ~65% of the maximal response.

DMPP behaved as a partial agonist with a maximal response equal to 65% of the response induced by the maximal concentration of ACh. Efficacy of DMPP for the α3β2β4α5 subunit combination did not match efficacies for other double and triple subunit combinations tested (table 1). S1 for DMPP (~45% of all sites) had an EC50 of 3.3 μM. S2 (~55% of all sites) had an EC50 of 110 μM. The Sα site detected by both ACh and DMPP differed in EC50 from those observed for these agonists on α3β2, α3β4, α3β2α5 and α3β4α5 AChRs.

In order to evaluate the yield of assembly of α5 into α3 AChRs expressed in oocytes, we immunoisolated [3H]-epibatidine labeled AChRs with subunit-specific mAbs (fig. 5). Precise evaluation of the composition of the AChRs formed in these conditions was constrained by the availability of mAbs. mAb210 crossreacts with both human α3 and human α5 AChR subunits (Wang et al., 1996). The efficiency of α5 subunit incorporation into α3 AChRs was estimated by an mAb 142 epitope-tagged α5 subunit (Wang et al., 1996) termed α5T. A specific mAb is not available for human β4 subunits.

Virtually all [3H]epibatidine binding sites were absorbed by the β2-specific mAb290 (Peng et al., 1994) from oocytes expressing α3, β2 and α5 subunits (fig. 5), and virtually none from oocytes expressing α3, β4 and α5 subunits (fig. 5). When all four subunits were expressed, more than 85% of the AChRs were found to contain β2 subunits. Efficiency of α5 coassembly with α3 and β2 subunits was 65%, and with α3 and β4 subunits was about 50% (fig. 5). When all four subunits were expressed, more than 70% of the AChRs contained α5 subunits (fig. 5). Hence, when all four (α3, β2, β4 and α5) AChR subunits are expressed in oocytes, the majority of AChRs contain α3, α5 and β2 subunits. The differences in expression levels of α3β2α5 (~10 fM/oocyte) and α3β4α5 (~2

Fig. 3. α5 subunits significantly increase Ca2+ permeability of α3β2 and α3β4 AChRs. Top and middle) Shift of the reversal potential of α5-containing and α5-less AChRs induced by a 10-fold increase of Ca2+ concentration from 1.8 to 18 mM. Representative currents induced by the application of voltage ramps to oocytes perfused by 100 μM ACh with 1.8 mM (dashed trace) or 18 mM Ca2+ (solid trace) in the extracellular solution are plotted against membrane potential. Currents induced by the ramps in agonist-free solutions are subtracted. Recordings were performed in Cl−-free solutions on oocytes preincubated in Cl−-free media (see “Materials and Methods”). Bottom) Plot of the reversal potential shifts induced by a 10-fold increase of extracellular Ca2+ concentration (from 1.8 to 18 mM) for muscle-type α1β1γδ and neuronal α7 AChRs (open bars), α3β2 and α3β4 (gray bars) and α3β2α5 and α3β2α5 AChRs (black bars). Averaged data were obtained from 7 to 14 oocytes as described on the top panel and represent mean ± S.E.
fM/oocyte) AChRs (fig. 5) did not allow for evaluation of efficiency of the incorporation of β4 subunits when all four subunits were expressed in oocytes.

**Analysis of subunit composition of native human AChRs using mAbs.** We used the available mAbs to assay incorporation of α5 subunits in AChRs from neuronal tissues of central and peripheral origin. The human neuroblastoma cell line SH-SY5Y expressing postsynaptic type α3 AChRs was used as a model of ganglionic type AChRs. Post-mortem human brain tissue from neocortex was used to characterize central and peripheral origin. The human neuroblastoma cell line SH-SY5Y expressing postsynaptic type α3 AChRs and for α4, β2 and α5 AChR subunits. Responses to consecutive applications of increasing concentrations of ACh to the oocytes voltage-clamped at −50 mV are displayed on the left. Traces shown were obtained from oocytes 3 days after injections of 10 ng of each cRNA. Bar and numbers above the traces mark duration of the application and concentration of the ACh. (Top right) Concentration-response curves for ACh and DMPP. Experimental data were fitted using the sum of two Hill equations yielding both high-affinity (S1) and low-affinity (S2) sites for both agonists. Hill coefficients were fixed to 1.5. Fit with one Hill equation resulted in Hill coefficient values below 0.7 for both curves. The ACh concentration-response curve had EC50 values of 24 ± 7 μM (S1, ~35% of all sites) and 344 ± 43 μM (S2, ~65% of all sites). The DMPP concentration-response curve had EC50 values of 24 ± 7 μM (S1, ~65% of all sites) and 344 ± 43 μM (S2, ~55% of all sites) with maximal currents reaching 65% of the current induced by 3 mM ACh. Data for ACh and DMPP were obtained from two different sets of oocytes and normalized as described for figure 4. (Bottom) Assembly of α3 AChRs evaluated by specific mAbs. Equal (10-ng) amounts of cRNAs for α3, β2, β4 and reporter epitope tagged α5 subunits were injected into oocytes in the combinations listed below the groups of bars on the graph. Aliquots of the oocyte extracts were immunodepleted extensively with mAb142-Actigel, which removed all the α5-containing AChRs, or with mAb290-Actigel, which removed all the β2-containing AChRs. By comparing the [3H]epibatidine binding sites in the extracts before and after adsorption with mAb142 or mAb210, we determined the efficiency of incorporation of α5 and β2 subunits into three- and four-subunit AChRs. Values represent the mean ± S.E. from at least three separate experiments.

AChRs that contain α5 or β4 subunits. As expected, no α4 AChRs were found.

Most (63%) of the human neocortex extract AChRs that contained β2 subunits also contained α4 subunits. Of these α4β2 AChRs, 36% may also contain α5 subunits because they could be adsorbed by mAb210.

In order to evaluate the relative amounts of various AChR subtypes in whole brain, we performed a similar immunoisolation of [3H]epibatidine binding sites from extracts of complete rat brains. As in human neocortex, the major [3H]epibatidine binding component was adsorbed by both mAb290 to α4 and
mAb290 to β2 (fig. 6), which confirms that α4β2 is the dominant central neuronal AChR with high affinity for epibatidine. About 20% of these α4β2 AChRs appeared to have α5 associated with them, because they could be preadsorbed with mAb210. The amount of α3 or α5 AChRs in this tissue was about 4% of the α4β2 AChRs. Most or all of these appeared to contain β2 subunits, but this measurement was difficult because so few α3 AChRs were present.

**Discussion**

Our results prove that, when expressed in *Xenopus* oocytes, human α5 subunits are efficiently incorporated with α3 and β2 or with α3 and β4 subunits to form AChRs that differ in both dose dependence of activation and cation channel properties from AChRs containing only α3 and β2 subunits or α3 and β4 subunits. These results suggest that α5 subunits alter channel properties because they contribute directly to structure and can alter the EC\(_{50}\) or efficacy of some agonists. Although they may not be part of the structure of the agonist binding sites, the α5 subunit contribution to the overall structure of the AChR influences the ability of the AChR to make the concerted changes in subunit orientation or conformation that are required for channel opening or desensitization.

It was shown recently that chick α5 subunits can efficiently assemble together with α4 and β2 subunits to form AChRs with distinct properties (Ramirez-Latorre et al., 1996). Immunoprecipitation studies have shown that only a minor fraction of native chick brain α4β2 AChRs contain α5 subunits (Conroy and Berg 1995). In contrast, a majority of native α3-containing AChRs, at least in autonomic ganglia, are thought to have α5 subunits incorporated (Conroy et al., 1992; Vernallis et al., 1993; Conroy and Berg 1995). Thus determination of the functional impact of α5 subunit on α3 AChRs is crucial to understanding the physiological contributions of individual subunits to native “ganglionic-type” neuronal nicotinic AChRs.

**Pharmacology.** When α5 is coexpressed with α3 and β2 subunits, two types of AChRs may be formed: α3β2 and α3β2α5. As we have shown previously by immune precipitation and have confirmed here, in these conditions more than 70% of the α3 AChRs contain α5 subunits (Wang et al., 1996). The presence of α5 subunits produces a uniform change in functional properties. Concentration-response curves for the α3β2α5 subunit combination do not resolve two subpopulations of AChRs. EC\(_{50}\) for ACh differs 50-fold between α3β2 and α3β2α5 AChRs. Additionally, the efficacy of DMPP changed dramatically between these two subunit combinations. DMPP had significantly higher efficacy (183%) than ACh for α3β2α5 AChRs. Higher efficacy of DMPP compared with ACh was reported previously for rat α3β2 and α3β4 AChRs expressed in the *Xenopus* oocytes (Cachelin and Jaggi, 1991). Oddly, however, when rat α3β4 AChRs were transiently expressed in HEK-293 cells, DMPP was reported to behave as a partial agonist with less than 30% efficacy compared with ACh (Wong et al., 1995). Overall, DMPP exhibited remarkable sensitivity to the human AChR subunit combination expressed. Despite only moderate changes in EC\(_{50}\) for the four α3 AChRs tested, DMPP exhibited large differences in efficacy. DMPP had only 13% efficacy for α3β4α5 AChRs, was as efficacious as ACh on α3β4 AChRs, was slightly more efficacious than ACh on α3β2 AChRs and was almost twice as efficacious as ACh on α3β4 AChRs. This characteristic of DMPP could prove useful in identification of the subunit composition of native human α3 AChRs.

Cytisine exhibited poor efficacy for all the human α3 AChRs tested. It had higher efficacy (50% for β4-containing AChRs than for β2-containing AChRs (20%). This difference in efficacy for cytisine between β2- and β4-containing AChRs was also observed for rat α3 AChRs (Papke and Heinemann, 1993). However, for rat α3β4 AChRs transiently expressed in the HEK-293 cells, cytisine behaved as a full agonist compared with ACh (Wong et al., 1995).

Of the four subunit combinations tested, concentration-response curves built for AChRs containing β4 subunits compared with AChRs containing β2 subunits were significantly steeper, with Hill coefficients closer to 2 for all agonists but cytisine. This could reflect the slower desensitization rates observed for β4-containing AChRs, which could permit better resolution of responses at high agonist concentrations. Alter-
natively, the presence of a subpopulation of AChRs with different agonist affinity could modify the slopes of concentration-response curves. Coverton et al. (1994) reported significantly higher Hill slopes in Xenopus oocytes for rat α3β4 AChRs than for α3β2 AChRs.

**Desensitization.** For both α3β2α5 and α3β4α5 AChRs, rates and magnitude of desensitization were higher than for α3β2 and α3β4 AChRs. Addition of the rat α5 subunit to α4β2 has also been reported to cause acceleration of desensitization (Ramirez-Latorre et al., 1996). Enhancement of desensitization in α5-containing AChRs might be expected to shift EC50 values for activation to higher concentrations. However, increases of apparent affinity for ACh and nicotine were observed when α5 subunits were added to α3β2 AChRs. Thus the pharmacological effects of α5 subunits probably do not reflect changes only in rates of desensitization.

Comparison of α3β2 and α3β4 AChRs indicates that switching of β2 for β4 structural subunits significantly influences both the kinetics and the pharmacological properties of the AChRs. Similar phenomena were described previously for heterologously expressed chick and rat α3-containing AChRs (Luetje and Patrick, 1991; Papke, 1993; Hussy et al., 1994; Gerzanich et al., 1995; Fenster et al., 1997). It was suggested that β2 and β4 subunits contribute directly to the ligand binding pocket on the interface with α subunits. This raises a question of the possible position of the α5 subunit in the α3 AChR pentamer and the mechanisms by which α5 might influence functional properties. Pentameric structure of α3 AChRs is assumed on the basis of homology within the gene family and from comparison of the sizes of AChRs obtained in sucrose-gradient experiments (Wang et al., 1996). The inability of α5 subunits to assemble directly with α3 or β subunits to form functional AChRs, together with lack of α5 influence on the ligand affinities in the equilibrium binding experiments (Wang et al., 1996) suggests that α5 subunits do not contribute to the ligand binding pocket at the interface between α3 and β subunits. This indicates that changes in the macroscopic kinetic properties and pharmacological profiles of α3β2α5 and α3β4α5 AChRs observed electrophysiologically are determined not by the α5 subunit's direct interaction with agonists but by the overall conformational changes that it induces in AChRs. In addition, an α5 subunit present in an AChR would be expected to contribute one-fifth of the amino acids lining the cation channel and thereby potentially affect ion flow directly.

**Ca**++ permeability and modulation. Native and recombinant α7 AChRs were shown to have Ca**++** permeability comparable to that of NMDA receptors (Bertrand et al., 1993; Seguela et al., 1993; Castro and Albuquerque, 1995). Previously it was shown that native and recombinant rat α3 AChRs have significant Ca**++** permeability (Fieber and Adams, 1991; Adams and Nutter, 1992; Vernino et al., 1992; Rogers and Dani, 1995). Dependence of the reversal potential on extracellular Ca**++** indicates that human α3β2 and α3β4 AChRs could conduct a significant amount of Ca**++** ions. Because of the much slower desensitization rates of α3 AChRs compared with α7 AChRs, α3 AChRs could potentially, over prolonged periods, conduct more Ca**++** than could α7 AChRs. Moreover, introduction of α5 subunits further increases the Ca**++** permeability of α3 AChRs, producing, after a 10-fold increase of extracellular Ca**++**, a shift of the reversal potential comparable to that of α7 AChRs. This suggests that α3α5β2 and α3α5β4 AChRs may play more important roles than previously suspected in ACh-induced Ca**++**-mediated effects in both the peripheral nervous system and the CNS.

Ca**++** permeability of neuronal AChRs is important because of the well-established role of Ca**++** influx in many physiological and pathophysiological processes. In autonomic ganglia, α3 AChRs are directly involved in synaptic transmission from preganglionic neurons. Ca**++** ions entering neurons through postsynaptic AChRs during EPSCs were shown to trigger a Ca**++**-dependent K+ current (Tokimasa and North, 1984). In the CNS, presynaptic nicotinic AChRs were shown to exert facilitatory effects by increasing presynaptic Ca**++** concentration (Mulle et al., 1992).

Potentiation by extracellular Ca**++** of recombinant and native AChRs is viewed as an important mechanism of modulation (Mulle et al., 1992; Vernino et al., 1992; Amador and Dani, 1995; Galzì et al., 1996). For chicken homomeric α7 AChRs, it was shown that divalent cation binding sites in extracellular domains are likely to mediate potentiation of the response by extracellular Ca**++**. It was proposed that Ca**++** potentiates responses by direct interaction with the nicotinic ligand binding site of the AChRs. Substitution of β2 for β4 subunits virtually eliminates Ca**++** potentiation of the human α3 AChR responses. This suggests that extracellular Ca**++** can modulate AChR function via “structural subunits” as well. Considering that the ligand binding pocket is formed by the interface of the α and β AChR subunits, a β2-located site of the domain responsible for the Ca**++** potentiation is not unexpected. Differential Ca**++** potentiation of the α3β2 and α3β4 AChRs could account for differences of Ca**++** flux observed for these AChRs recombinantly expressed in HEK-293 cells (Mahaffy et al., 1996).

**Recombinant and native α3 AChRs.** As shown by Conroy and Berg (1995) on neurons of chick ciliary ganglia, immunoprecipitation and immunoblot analysis strongly suggests that at least a portion of α3 AChRs contain four kinds of subunits: α3, β2, β4 and α5. Coexpression of the corresponding human subunits in Xenopus oocytes resulted in functional AChRs with a distinct concentration-response curve for ACh. Hill equation fit indicated at least two populations of AChRs. One population (55%–65% of the total) had significantly lower affinity for ACh (EC50 = 345 μM) and DMPP (EC50 = 110 μM) compared with the other subunit combinations tested (table 1), which suggests that it might result from the combination of four kinds of subunits. The higher-affinity site had affinities for both ACh and DMPP close to the values for α3β2 AChRs. The distribution of affinities for ACh estimated for oocytes expressing all four subunits indicates that the contribution of α3β2α5 AChRs to the mixture of AChRs expressed was negligible. Immune precipitation analysis showed that greater than 70% of the α3 AChRs contained both α5 and β2 subunits. This strongly suggests that the population of α3 AChRs with unusually low affinity for ACh contains all four subunits. Overall, data on immunoidentification confirm not only the high efficiency of coassembly of α5 subunits with α3 and β2 or with α3 and β4 AChR subunits as previously determined (Wang et al., 1996) but also indicate the incorporation of α5 subunits into α3 AChRs containing both β2 and β4 subunits.

Examination of AChR subunit expression in human neocortex confirmed that α4β2 AChRs are the dominant non-
Functional Role of α5 Subunit in α3 AChRs

Aνbungarotoxin binding neuronal AChR in the brain (Whiting and Lindstrom, 1986, Flores et al., 1992). A significant part (up to 25%) of human neocortex α4-containing AChRs could be immunodepleted by preadsorption with mAb 210, which binds to both α3 and α5 subunits. The amount of mAb210-immunodepleted α4 containing AChRs appears to be larger than the amount of AChRs that could be immunosolated from neocortex by mAb210 alone. This discrepancy might be in part due to degradation of the AChRs during the day required for the additional step of immunodepletion. A majority of the AChRs that bind to mAb210 could be depleted by the α4-specific mAb299. These data strongly suggest that the α5 subunit is incorporated in some α4β2 AChRs, although incorporation of α3 or of some other unknown AChR subunit that has affinity for mAb210 could not be excluded. According to the in situ hybridization studies, expression of α3, that of α4 and that of α5 have different but overlapping patterns in mammalian brain. Cerebral cortex contains messages for all of these AChR subunits as well as for β2 subunits (Deneris et al., 1991).

As expected, ganglionic-type neurons from the human neoblastoma cell line SH-SY5Y were found to have a significantly different pattern of AChR subunit expression. α3α5 subunit-containing AChRs account for all of the high-affinity [3H]epibatidine binding sites in these cells, with no detectable expression of α4 subunits. Half of these α3a5 AChRs contain β2 subunits, β4 subunits probably substitute for β2 in the rest of the AChRs.

Comparison of the data on AChR subunit expression in the human neocortex with the data obtained from the rat total brain extract reveals significant differences in levels of expression of mAb210 binding AChRs. A small but significant part of the α4-containing AChRs from the rat brain could be immunodepleted by binding to mAb210. The overall level of α3 and α5 AChR subunits is very small (~4%) relative to β2 and α4 subunits, a level much lower than in the human neocortex. These differences could result from differences in the origin of the brain tissue, with human cortex representing only its local distribution of the AChRs. Alternatively, differences in expression could be interpreted as due to differences between rats and humans.

Unlike message for the α4 AChR subunits, which has a rather diffuse and diverse pattern of expression in the verbranebrain, the patterns of α3 and α5 subunit expression are much more localized. α5 subunit mRNAs are present at modest levels in the cortex, at higher levels in the interduncular nucleus and at the highest levels in the ventral tegmental area and substantia nigra pars compacta (Wada et al., 1989; Boulter et al., 1990). These areas include regions in which there is nicotinic facilitation of dopamine release. Recently it has been suggested (Le Novere and Changeux, 1995) that some of the regions thought to contain α3 on the basis of the in situ hybridization studies actually contain the closely related but pharmacologically distinct α6 subunit (Gerzanich et al., 1997). This prediction has been confirmed immunohistochemically (Goldner et al., 1997).

Pharmacological, kinetic and Ca++-dependent effects of α5 subunits on α3 AChRs imply that α5 subunits could be utilized effectively for fine-tuning neuronal nicotinic AChR function in vivo. In the periphery, synaptic α3 AChRs from human autonomic neurons are the most likely to be functionally affected by the presence of α5 AChR subunits. In the human brain, α5 subunits may be associated with a small fraction of α4β2 AChRs as well as with α3 AChRs.

References


Cachelin AB and Jaggi R (1991) f subunits determine the time course of desensitization in rat α3 neuronal nicotinic acetylcholine receptors. Pflügers Arch 14:579–582.


Send reprint requests to: Dr. Jon Lindstrom, 217 Stemmler Hall, 36th and Hamilton Walk, Philadelphia, PA 19104-6074.