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Mapping of Ligand Binding Sites of Neuronal Nicotinic Acetylcholine Receptors Using Chimeric α Subunits

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SUMMARY

We constructed a series of chimeric neuronal nicotinic acetylcholine (ACh) receptor (nAChR) α subunits to map the location of amino acid residues that determine the pharmacological properties of these receptors. The α2 and α3 subunits form pharmacologically distinct nAChRs upon expression, in combination with the β2 subunit, in *Xenopus* oocytes. The α2β2 subunit combination is insensitive to the nicotinic antagonist neuronal bungarotoxin (NBT) and is much more sensitive to nicotine than to ACh. In contrast, the α3β2 subunit combination is potently inhibited by NBT and is much less sensitive to nicotine than to ACh. Chimeric subunits were constructed by replacing portions of α2 or α3 with the analogous portion of the other α subunit. Pharmacological analysis of receptors formed by these chimeric subunits, in combination with β2, revealed that amino acid residues involved in determining NBT sensitivity were located within sequence segments 84-121, 121-181, and 195-215. Amino acid residues that determine agonist sensitivity were located within sequence segments 1-84 and 195-215. Within region 195-215, we used site-directed mutagenesis to demonstrate the importance of Gln-198 of α3 (proline in α2) in determining both the antagonist sensitivity and the agonist sensitivity of neuronal nAChRs.

nAChRs are found at the neuromuscular junction and throughout the central and peripheral nervous systems. Neuronal nAChR subunits include α2, α3, α4, α5, α6, α7, α8, β2, β3, and β4 (1–5). These subunits have been shown, using the *Xenopus* oocyte expression system, to associate in various combinations to form physiologically and pharmacologically distinct receptors (6–9). Characterization of nAChRs in a variety of neuronal preparations reveals not only that neuronal nAChRs differ physiologically and pharmacologically from muscle nAChRs (10) but also that multiple neuronal nAChR subtypes exist *in vivo* (1, 2, 11). Differential subunit association is suggested as the mechanism for generating this diversity by the results of experiments in *Xenopus* oocytes and by experiments with cultured neurons (12).

The nature of the neurotransmitter binding site of nAChRs has been the subject of intense investigation for many years. On the muscle-type nAChRs, the binding sites for cholinergic ligands have long been thought to reside solely on the α subunits. Indeed, the use of affinity labeling techniques has identified amino acid residues Tyr-93, Trp-149, Tyr-190, Cys-192, Cys-193, and Tyr-198 of the *Torpedo* α subunit as being at or near the ACh binding site of the receptor (13–17). Recent work has shown the structure of cholinergic binding sites to be more complex, with contributions from both the γ and δ subunits (17–20). The ligand binding sites of neuronal nAChRs appear to be similarly complex, because both α and β subunits make contributions to the pharmacological properties of these receptors (6, 8, 9).

In this study, we investigate the structural basis for pharmacological diversity among neuronal nAChRs. The amino acid residues of the *Torpedo* α subunit that affinity labeling experiments have located at the ligand binding site are conserved in both muscle and neuronal α subunits. These residues may form portions of the ligand binding site common to all nAChRs but are unlikely to be responsible for the pharmacological diversity observed among members of this receptor family. Our experimental approach is designed to identify amino acid residues that confer this pharmacological diversity. We have constructed a series of chimeric receptor subunits consisting of portions of two pharmacologically distinct α subunits, α2 and α3. Using the *Xenopus* oocyte expression system to determine the pharmacological properties of receptors formed by these chimeric subunits, we have mapped the location of amino acid residues that confer pharmacological diversity. We then examined the roles of individual amino acid residues by site-directed muta-

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ABBREVIATIONS: nAChR, nicotinic acetylcholine receptor; ACh, acetylcholine; NBT, neuronal bungarotoxin; AchE, acetylcholinesterase; DDF, *p*-dimethylaminobenzenediazonium fluoroborate; HEPES, *N*-(2-hydroxyethyl)piperazine-*N*′-(2-ethanesulfonic acid).

genesis. Although both α and β subunits make substantial contributions to the pharmacological properties of neuronal nAChRs, in this study we focus on the α subunit contribution. Consequently, all receptors are formed with the same β subunit (β2).

Experimental Procedures

Materials. *Xenopus laevis* frogs were purchased from Nasco. RNA transcription kits were from Stratagene. Diguanosine triphosphate was from Pharmacia. SP6 polymerase and RNasin were from Promega. ACh, nicotine, atropine, 3-aminobenzoic acid ethyl ester, and type I collagenase were from Sigma. NBT (toxin F) was a gift from Dr. Richard Zigmond (Department of Neuroscience, Case Western Reserve University, Cleveland, OH).

Mutagenesis and construction of chimeric receptors. The α2 and α3 subunits are represented schematically in Fig. 1. Chimeric subunits were constructed using naturally occurring and mutagenically installed restriction enzyme cleavage sites. Our notation for these chimeric subunits is to list the source of the amino-terminal portion followed by the point in the amino acid sequence where the joint is made and then by the source of the carboxyl-terminal portion. For example, the chimeric subunit α3-195-α2 is composed of α3 sequence from the amino terminus until residue 195, after which it is composed of α2 sequence. Restriction sites were installed in the cDNAs encoding α2 and α3 by site-directed mutagenesis, using a Muta-Gene mutagenesis kit (Bio-Rad). The *Bgl*II site at Lys-84, the *Nae*I site at Pro-121, the *Sma*I site at Ala-181, and the *Bgl*II site at Glu-195 are all naturally occurring sites in the α3 cDNA, whereas the *Ase*I site at Ile-215 was installed by mutagenesis. The *Bgl*II site at a position in the α2 cDNA analogous to Glu-195 of α3 is naturally occurring in the α2 cDNA, whereas the *Bgl*II site (position analogous to Lys-84 of α3), the *Nae*I site (position analogous to Pro-121 of α3), the *Sma*I site (position analogous to Ala-181 of α3), and the *Ase*I site (position analogous to Ile-215 of α3) were installed by mutagenesis. Mutants were selected by DNA sequencing. The coding regions of all mutant constructs were sequenced in their entirety to confirm that no unintended mutations had occurred. We used the double-stranded sequencing protocol of Kraft *et al.* (21). When restriction enzyme cleavage sites were installed, the original amino acid sequence was either maintained or changed to that of the other subunit (α2 or α3). Thus, chimeric subunits could be constructed consisting of a portion of α2 and a portion of α3, with no novel amino acids introduced. Chimeric subunits were verified by sequencing across the junctions. Nonfunctional chimeras (see below) were sequenced across the chimeric junctions and were analyzed by restriction enzyme digestion. The nonfunctional chimeric constructs did not differ from their functional parental sequences.

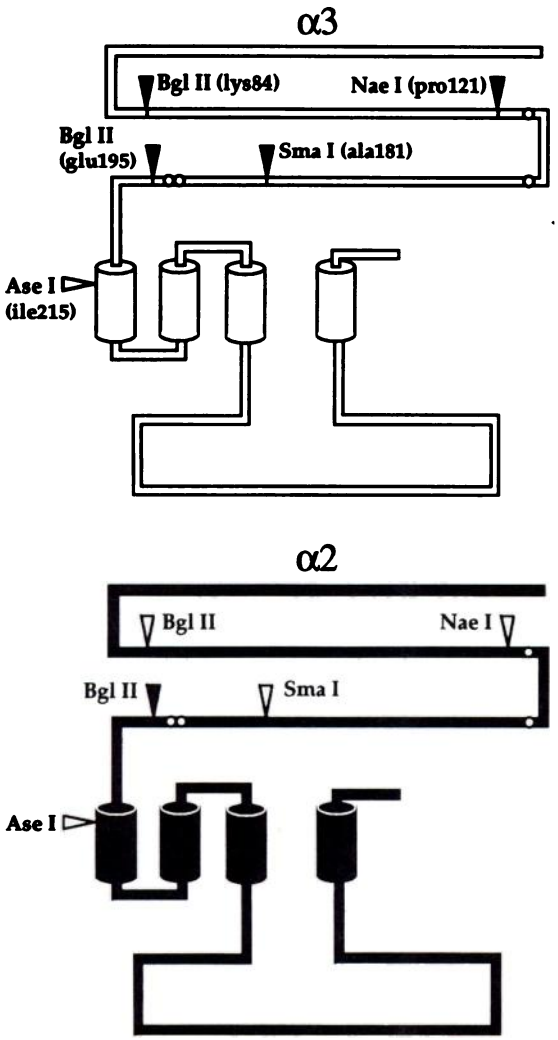

Fig. 1. Chimeric subunits were constructed using both naturally occurring and mutagenically installed restriction enzyme cleavage sites. The transmembrane orientation of neuronal nAChR subunits is presented schematically. For each subunit, the large amino-terminal extracellular domain is shown at the *top*. *Cylinders*, putative transmembrane domains. *Circles*, locations of cysteine residues 128, 142, 192, and 193. *Arrowheads*, locations of restriction sites. The corresponding position in the amino acid sequence is shown in *parentheses*. *Black arrowheads*, naturally occurring restriction sites; *white arrowheads*, mutagenically installed restriction sites. All numbering of amino acid positions in this study refers to the mature α3 amino acid sequence.

The α2 and α3 cDNAs were inserted into the Bluescript SK− vector (Stratagene). This allowed helper phage-mediated single-stranded DNA rescue as needed for the mutagenesis protocol and would allow *in vitro* RNA transcription from the mutant and chimeric constructs. However, when we injected RNA transcribed from the α2, α3, α2-195-α3, or α3-195-α2 Bluescript SK− constructs in combination with β2 RNA, no receptor expression was detected. This occurred with several lots of RNA and several sets of oocytes. We tried using 7-methyl-diguanosine triphosphate to cap the transcripts, instead of diguanosine triphosphate. Also, because all of the constructs were made to use the T3 promoter for RNA transcription, we reversed the orientation of the α2 insert, to use the T7 promoter. Neither of these strategies resulted in expression of functional subunits. We then compared RNA made from the original α2 in pSP65, RNA made from α2 in Bluescript SK−, and RNA made from α2 cut out of Bluescript SK− and reinserted in pSP65. RNA transcribed from α2 in Bluescript SK− was again unable to direct the expression of functional subunits. However, when RNA made from α2 cut out of Bluescript SK− and reinserted in pSP65 was injected into oocytes, functional expression occurred. We sequenced the 5′ end of the inserts as well as the chimeric joints of α2-195-α3 and α3-195-α2 but found no obvious problems. This work encompassed the use of eight different sets of oocytes and five different lots of RNA. In no case was RNA transcribed from one of these Bluescript SK− constructs able to cause functional expression. This is unusual because RNA made from a mutant β2 in Bluescript SK− for a different set of experiments worked well. In the end, we were unable to determine the source of the problem and decided to transfer all mutant and chimeric subunit cDNA inserts back to the pSP64 and pSP65 RNA transcription vectors (Promega).

RNA transcribed from 12 of 15 chimeric and mutant cDNA constructs in pSP64–65 was able to direct the expression of functional receptor subunits. RNA transcribed from three of the chimeric DNA constructs (α3-84-α2, α3-121-α2, and α3-181-α2) was unable to direct the expression of functional receptor subunits. For α3-181-α2 we tried two different lots of RNA and two different batches of oocytes. For α3-84-α2 we tried two different lots of RNA prepared from two independent cDNA constructs, as well as three different batches of oocytes.

Sequencing across the chimeric junction and restriction enzyme digestion showed no unintended alteration of nucleotide sequence. Thus, we could find no technical reasons for the inability of RNA transcribed from these cDNA constructs to direct expression of functional receptors. We do not, at present, know whether these chimeric subunit proteins are actually made in the oocytes.

Injection of *in vitro* synthesized RNA into *Xenopus* oocytes. Diguanosine triphosphate-capped RNA was synthesized *in vitro* from linearized template DNA encoding the α2, α3, and β2 subunits, as well as the various chimeric and mutant subunits, as described previously (22), by using an RNA transcription kit, RNasin, and SP6 polymerase. Mature *X. laevis* frogs were anesthetized by submersion in 0.1% 3-aminobenzoic acid ethyl ester and oocytes were surgically removed. Follicle cells were removed by treatment with type I collagenase for 2 hr at room temperature. Each oocyte was injected with a total of 10 ng of RNA in 50 nl of water and was incubated at 19° in modified Barth's saline (88 mM NaCl, 1 mM KCl, 2.4 mM $NaHCO_3$, 0.3 mM $CaNO_3$, 0.41 mM $CaCl_2$, 0.82 mM $MgSO_4$, 100 μg/ml gentamicin, 15 mM HEPES, pH 7.6) for 2–7 days. The molar ratio of RNAs encoding each subunit was 1:1.

Electrophysiological recordings. Oocytes were perfused at room temperature (20–25°) in a 300-μl chamber with perfusion solution (115 mM NaCl, 1.8 mM $CaCl_2$, 2.5 mM KCl, 10 mM HEPES, pH 7.2, 1 μM atropine). Perfusion was continuous at a rate of approximately 20 ml/min. Agonists were diluted in perfusion solution, and the oocytes were exposed to agonist for approximately 10 sec, using a solenoid valve. NBT sensitivity was tested by comparing ACh-induced current responses before and after the oocytes were incubated for 30 min in perfusion solution containing various concentrations of NBT and 100 μg/ml bovine serum albumin. Preincubation with NBT results in a slowly reversible competitive blockade of α3β2 but not α2β2 (8, 22, 23). In addition, coapplication of NBT with agonist reveals a rapidly reversible competitive blockade of subunit combinations other than α3β2.[2] Our experimental protocol incorporates a 5-sec wash after NBT incubation and before measurement of the postincubation ACh response. This wash step eliminates the rapidly reversible blockade of subunit combinations other than α3β2. Thus, our protocol detects only slowly reversible blockade by NBT. Under these conditions, the large difference in sensitivity to NBT provides a powerful tool with which to compare the α2 and α3 subunits and their chimeras.

Current responses to agonist application were studied under two-electrode voltage-clamp at a holding potential of −70 mV. Experiments were performed using an Axoclamp 2A voltage-clamp unit (Axon Instruments). Micropipettes were filled with 3 M KCl and had resistances of 0.5–3.0 MΩ. Agonist-induced responses were filtered at 15 Hz (eight-pole Bessel filter; Frequency Devices) and then captured, stored, and analyzed on a Macintosh IIci computer using a data acquisition program written with LabVIEW (National Instruments) and LIB I (University of Arizona) software.

Dose-response data were fit with Passage II software by the nonlinear least squares method, using the equation current = maximum current/$[1 + (EC_{50}/[\text{agonist}]^n)]$, where n and EC_{50} represent the Hill coefficient and the agonist concentration producing half-maximal response, respectively. These dose-response curves must be considered crude approximations. Rapid extensive desensitization of nAChRs occurs at high agonist concentrations. The size and shape of *Xenopus* oocytes make rapid application of agonists difficult, and thus determination of the true peak response to high concentrations of agonist is problematic. Because this makes the maximal response an unreliable standard with which to normalize data, we have normalized agonist responses to the response of the same oocyte to a low concentration of ACh. For α2β2 and α3β2, as well chimeric and mutant receptors displaying EC_{50} values for ACh similar to those of α2β2 and α3β2, this concentration was 1 μM. For α2-84-α3β2, α2-121-α3β2, and α2-181-α3β2, the concentration was 100 nM, 10 nM, and 1 nM, respectively.

Alteration of the α to β RNA injection ratio. We previously investigated whether alteration of the ratio of RNAs encoding α and β subunits that were injected into oocytes might alter the pharmacological properties of the expressed receptors. Detailed examination of the α4β4 subunit combination showed no change in pharmacological properties upon variation of the α to β subunit RNA ratio from 1:4 to 4:1 (9). In the present study we examined this issue again. The α3β2 and α2β2 subunit combinations were expressed by injecting oocytes with α and β RNA at molar ratios of 1:4, 1:1, and 4:1. The NBT sensitivity of α3β2 and α2β2 was not affected by alteration of the α to β RNA injection ratio (data not shown).[3] Also, alteration of the α to β RNA injection ratio had no significant effect on the sensitivity of α3β2 to ACh and nicotine (data not shown). When the ratio of α2 to β2 subunit RNA was varied from 1:1 to 4:1, no change was seen in the sensitivity of the expressed receptor to ACh and nicotine. However, when α2β2 was expressed using an α2 to β2 RNA injection ratio of 1:4, the difference in effectiveness between nicotine and ACh was greatly reduced and was no longer statistically significant. This effect was found to be attributable to one particular lot of α2 RNA injected in combination with one particular lot of β2 RNA. Receptors expressed from these RNA lots, at an α2 to β2 ratio of 1:4, were slightly less sensitive to nicotine than to ACh. When different lots of RNA were used, changing the ratio of α2 to β2 subunit RNA from 1:1 to 1:4 had no effect on the sensitivity of the expressed receptors to ACh and nicotine.

Although the alteration of α2β2 pharmacological properties occurred only with one set of RNA lots and we did not use these RNA lots to generate data, we felt that this result had the potential to complicate some of our agonist sensitivity experiments. The nicotine/ACh ratio of α2β2 expressed in this case was slightly less than 1. This is similar to the nicotine/ACh ratio displayed by receptors formed by the α2-84-α3, α2-121-α3, α2-181-α3, and α2-195-α3 chimeric subunits (see Fig. 5A) and by the α2, D191N,A194E,P198Q and α2,P198Q mutant subunits (see Fig. 6). Although receptors formed by these chimeric and mutant subunits were expressed by injecting RNA in an α to β ratio of 1:1, it is possible that whatever altered the pharmacological properties of the α2β2 "1:4" receptors occurred more readily with the receptors formed by these chimeric and mutant subunits. To rule out this possibility, we expressed receptors by injecting RNA encoding one of three different chimeric subunits (α2-121-α3, α2-195-α3, or α3-195-α2) or one of two different mutant subunits (α2,D191N,A194E,P198Q or α2,P198Q), in combination with β2, at an α to β ratio of 4:1. If these receptors can exist in two forms, then it would be expected that injecting RNA at an α to β ratio of 4:1 would shift the nicotine/ACh ratio to a higher value. This did not happen. In no case did we observe a change in the nicotine/ACh ratio (data not shown). This suggests that the shifted nicotine/ACh ratio of receptors formed by these chimeric and mutant subunits is intrinsic to the subunits and not due to changes in subunit stoichiometry.

Statistical analysis. Statistical significance was determined by using a two-sample *t* test after an *F* test to ensure equality of variance. For samples with unequal variance ($p > 0.05$), statistical significance was determined by using a two-sample *t* test for samples with unequal variance (Cochran's method).

Results

Formation of pharmacologically distinct receptors by α2 and α3. The α2 and α3 subunits can each form functional neuronal nAChRs when expressed in *Xenopus* oocytes in combination with the β2 subunit. These two subunit combinations differ in their sensitivity to antagonists and agonists (8, 9). The α2β2 subunit combination was unaffected by incubation with a 100 nM concentration of the antagonist NBT (Fig. 2A, *left traces*). In contrast, the α3β2 subunit combination was com-

[2] R. Papke, personal communication.

[3] K. Poth and C. Luetje, unpublished observations.

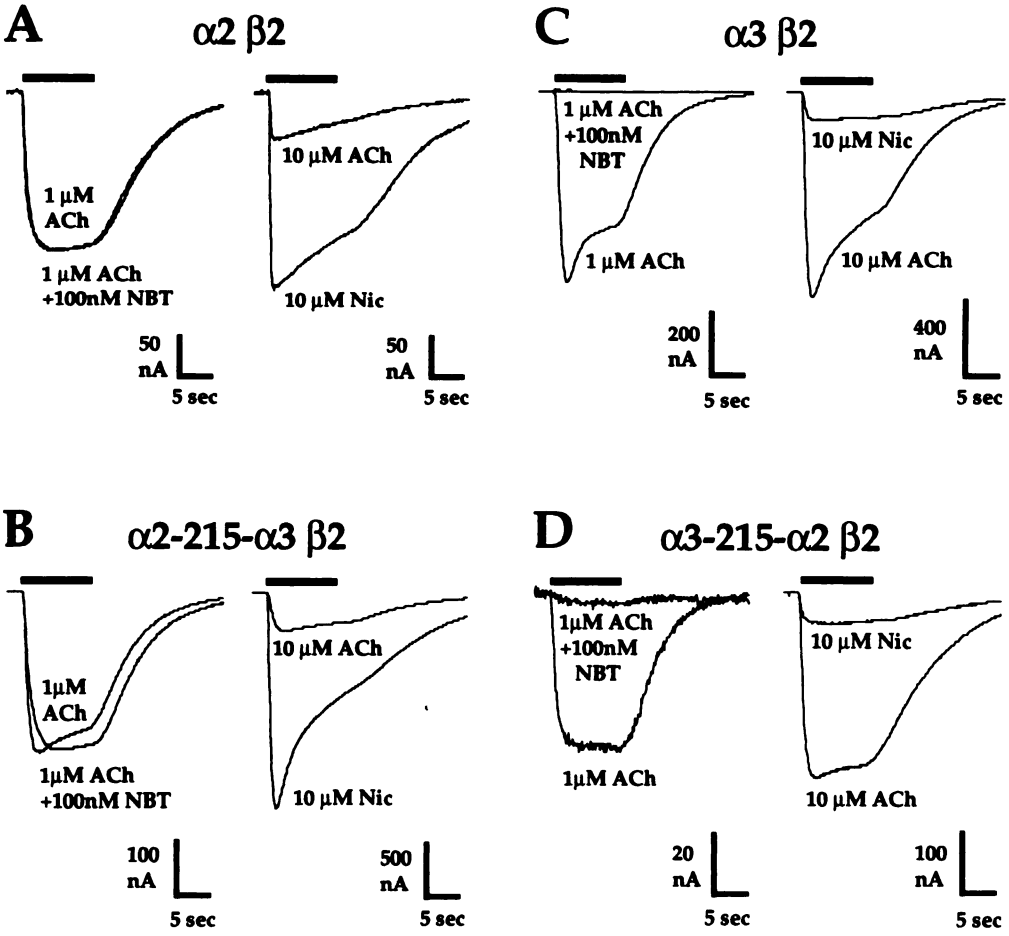

Fig. 2. The pharmacological properties of $\alpha 2$ and $\alpha 3$ can be transferred from one subunit to another by transfer of the amino-terminal extracellular domain. A, $\alpha 2\beta 2$ is not blocked by NBT and is more sensitive to nicotine (*Nic*) than to ACh. *Left set of traces*, current responses of an $\alpha 2\beta 2$-expressing oocyte before and after a 30-min incubation with 100 nM NBT. *Right set of traces*, current responses of a different $\alpha 2\beta 2$-expressing oocyte to 10 μM ACh and 10 μM nicotine. Note that the magnitude of the response to 10 μM ACh on the *right* is less than the magnitude of the response to 1 μM ACh on the *left*. This is due to the fact that the left and right sets of traces are from different oocytes, obtained from different frogs, injected at different times with different lots of RNA. This difference demonstrates the variability in expression level encountered in oocyte expression studies. B, The chimeric subunit containing the first 215 amino acid residues of $\alpha 2$ forms receptors with pharmacological properties similar to those of $\alpha 2\beta 2$. *Left set of traces*, current responses of an $\alpha 2$-215-$\alpha 3\beta 2$-expressing oocyte before and after a 30-min incubation with 100 nM NBT. *Right set of traces*, current responses of another $\alpha 2$-215-$\alpha 3\beta 2$-expressing oocyte to 10 μM ACh and 10 μM nicotine. C, $\alpha 3\beta 2$ is blocked by NBT and is less sensitive to nicotine than to ACh. *Left set of traces*, current responses of an $\alpha 3\beta 2$-expressing oocyte before and after a 30-min incubation with 100 nM NBT. *Right set of traces*, current responses of another $\alpha 3\beta 2$-expressing oocyte to 10 μM ACh and 10 μM nicotine. D, The chimeric subunit containing the first 215 amino acid residues of $\alpha 3$ forms receptors with pharmacological properties similar to those of $\alpha 3\beta 2$. *Left set of traces*, current responses of an $\alpha 3$-215-$\alpha 2\beta 2$-expressing oocyte before and after a 30-min incubation with 100 nM NBT. *Right set of traces*, current responses of another $\alpha 3$-215-$\alpha 2\beta 2$-expressing oocyte to 10 μM ACh and 10 μM nicotine.

pletely blocked by 100 nM NBT (Fig. 2C, *left traces*). The $\alpha 2\beta 2$ and $\alpha 3\beta 2$ subunit combinations also differ in their sensitivity to agonists. The $\alpha 2\beta 2$ subunit combination is much more sensitive to nicotine than to ACh (Fig. 2A, *right traces*). In contrast, the $\alpha 3\beta 2$ subunit combination is much less sensitive to nicotine than to ACh (Fig. 2C, *right traces*). These results demonstrate that, although the $\alpha 2$ and $\alpha 3$ subunits are homologous (57% amino acid sequence identity), receptors formed by these subunits are pharmacologically distinct. To generate a map of the sequence segments responsible for this pharmacological diversity, we have exploited the homology between these two proteins to construct a series of chimeric receptor subunits composed of portions of $\alpha 2$ and $\alpha 3$. The $\alpha 2$ and $\alpha 3$ subunits are represented schematically in Fig. 1. The installation of restriction enzyme cleavage sites and the construction of chimeric subunits is discussed in detail in Experimental Procedures.

Evidence that the pharmacological properties of neuronal nAChR α subunits are conferred by the amino-terminal extracellular domain. Initially we wanted to determine whether the amino acids that confer the specific pharmacological properties of neuronal nAChR α subunits are located within the sequence segment preceding the first transmembrane domain. This sequence segment is thought to comprise the extracellular domain of each subunit. We constructed chimeric subunits consisting of the first 215 amino acid residues of one subunit followed by the remaining amino acid residues of the other subunit ($\alpha 2$-215-$\alpha 3$ and $\alpha 3$-215-$\alpha 2$). The pharmacological properties of receptors formed by these chimeric subunits were determined upon expression (in combination with $\beta 2$) in *Xenopus* oocytes.

Fig. 2 compares the pharmacological properties of receptors formed by the chimeric subunits with those of receptors formed

by wild-type α2 or α3. The subunit containing the amino-terminal portion of α2 (α2-215-α3) (Fig. 2B) formed receptors with the same pharmacological properties as the wild-type α2β2 receptor (Fig. 2A), i.e., more responsive to nicotine than to ACh and not blocked by incubation with 100 nM NBT. The subunit containing the first 215 amino acid residues of α3 (α3-215-α2) (Fig. 2D) formed receptors with the same pharmacological properties as the wild-type α3β2 receptor (Fig. 2C), i.e., less responsive to nicotine than to ACh and blocked by 100 nM NBT. Thus, both agonist sensitivity and antagonist sensitivity segregate with the amino-terminal extracellular domain of the α subunits.

Mapping of regions of α subunits involved in NBT sensitivity. We produced a more detailed map of the location of pharmacologically important amino acid residues by constructing a series of chimeric subunits joined at amino acid positions 84, 121, 181, and 195. Receptors formed by each of these subunits (in combination with β2) were then tested for sensitivity to 100 nM NBT. Fig. 3A shows the NBT sensitivity of receptors formed by chimeric subunits consisting of an amino-terminal portion of α2 followed by a carboxyl-terminal portion of α3. The α3β2 subunit combination, (Fig. 3A, *top*) was almost completely inhibited by incubation with 100 nM NBT (99.1 ± 0.6% inhibition). In contrast, the α2β2 receptor (Fig. 3A, *bottom*) was completely resistant to 100 nM NBT. Replacement of the first 84 amino acid residues of α3 with α2 sequence had a small (but significant, $p < 0.05$) effect on NBT sensitivity; receptors formed by α2-84-α3 were almost as sensitive to NBT blockade (92.8 ± 3.8% inhibition by 100 nM NBT) as were receptors formed by the wild-type α3 subunit. This result suggests that amino acid residues between positions 1 and 84 of α3 are of no more than minor importance in determining NBT sensitivity. Further replacement of α3 sequence with α2 sequence between positions 84 and 121 (α2-121-α3) resulted in a loss of NBT sensitivity (49.6 ± 8.3% inhibition by 100 nM NBT). Replacement of the first 181 amino acid residues of α3 with α2 sequence (α2-181-α3) resulted in a complete loss of NBT sensitivity. Because loss of NBT sensitivity occurred upon replacement of α3 sequence between positions 84 and 121 and between positions 121 and 181, we conclude that these two sequence segments of α3 contain amino acid residues that are involved in determining NBT sensitivity.

Fig. 3B shows the NBT sensitivity of receptors formed by chimeric subunits that are the mirror image of those in Fig. 3A. These chimeric subunits consist of an amino-terminal portion of α3 followed by α2 sequence. Unfortunately, we were unable to demonstrate functional expression of the subunits α3-84-α2, α3-121-α2, or α3-181-α2 (see Experimental Procedures). The chimeric subunit in which the first 195 amino acid residues of α2 were replaced with α3 sequence (α3-195-α2) formed functional receptors that were insensitive to 100 nM NBT. This subunit contains regions of α3 (sequence segments 84-121 and 121-181) shown to be important for NBT sensitivity (Fig. 3A). The fact that receptors formed by this subunit are not blocked by NBT demonstrates that, whereas sequence segments 84-121 and 121-181 are necessary for NBT sensitivity, they are not sufficient to confer it on the α2 subunit. Only when the α2 sequence segment from position 195 to 215 was replaced by α3 sequence, to form α3-215-α2, was NBT sensitivity achieved (91.6 ± 3.7% inhibition by 100 nM NBT). Similarly, the insensitivity to NBT of receptors formed by α2-181-α3 and α2-195-α3 demonstrates that region 195-215 of α3 may be necessary, but is not sufficient, to confer NBT sensitivity. Thus, amino acid residues within three distinct sequence segments of α3 are required to confer NBT sensitivity.

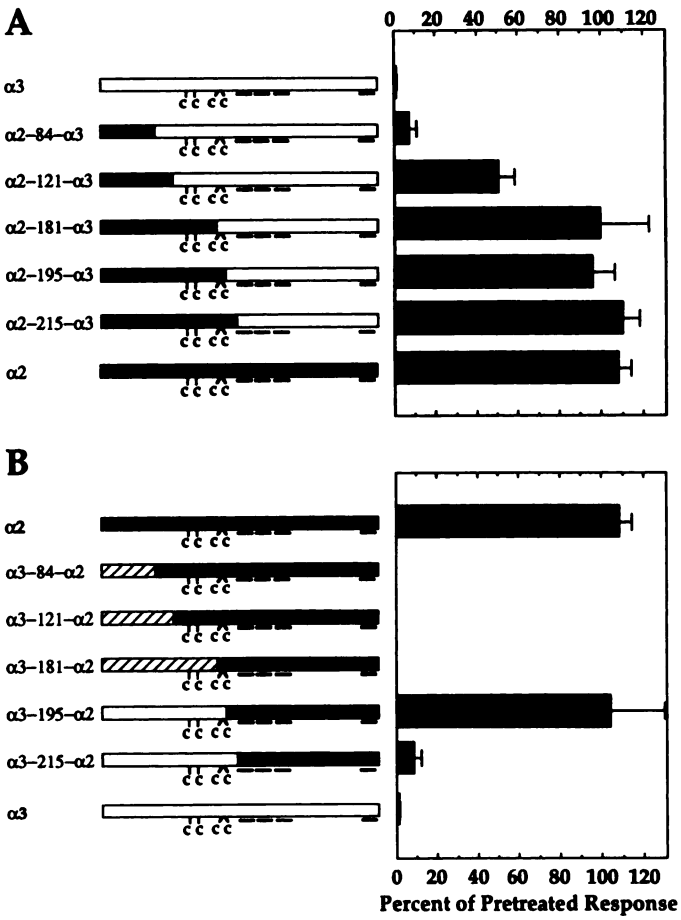

Fig. 3. Amino acid residues involved in the NBT sensitivity of α3 are located within sequence segments 84-121, 121-181, and 195-215. *Left*, schematic representations of the chimeric subunits tested, showing the locations of important cysteines (c) and the putative transmembrane domains (*bars*). The amino terminus is to the *left*. *White*, sequence derived from α3; *black*, sequence derived from α2. *Right*, NBT sensitivity of receptors formed by each chimeric subunit in combination with β2. ACh-induced current responses were measured before and after a 30-min incubation with 100 nM NBT. A concentration of ACh below the EC_{50} was used to test NBT sensitivity (usually 1 μM). In several cases, however, it was necessary to use a lower concentration of ACh (see Fig. 7 and accompanying text). The post-treatment response is presented as a percentage of the pretreatment control response (mean ± standard deviation of three separate oocytes). Significantly different from α3 are α2-84-α3 ($p < 0.05$), α2-121-α3 ($p < 0.001$), α2-181-α3 ($p < 0.01$), α2-195-α3 ($p < 0.001$), α2-215-α3 ($p < 0.001$), α3-195-α2 ($p < 0.01$), α3-215-α2 ($p < 0.05$), and α2 ($p < 0.001$). Significantly different from α2 are α2-84-α3 ($p < 0.001$), α2-121-α3 ($p < 0.001$), and α3-215-α2 ($p < 0.001$). A, NBT sensitivity of receptors formed by each of a series of chimeric subunits in which increasingly large portions of the amino-terminal end of α3 were replaced by the corresponding portion of α2. B, NBT sensitivity of receptors formed by each of a series of chimeric subunits in which increasingly large portions of the amino-terminal end of α2 were replaced by the corresponding portion of α3. *Hatch marks* on chimeric subunits α3-84-α2, α3-121-α2, α3-181-α2 are to indicate that injection of RNA encoding each of these subunits, in combination with RNA encoding β2, does not result in the expression of functional receptors.

Importance of the amino acid residue at position 198 for NBT sensitivity. We examined the sequence segment

between residues 195 and 215 in more detail by making changes at the amino acid level (Fig. 4). This region is highly conserved, with 15 of 20 amino acids being identical in the two subunits. Of the five amino acid differences, position 198 was of particular interest. In $\alpha 3$, this residue is a glutamine, whereas at the analogous position of $\alpha 2$ this residue is a proline. The presence or absence of a proline at this position might have a large effect on the structure of the binding site. To test the role of a residue between positions 195 and 215, it was necessary to include the other sequence segments shown to be important in determining NBT sensitivity, i.e., 84-121 and 121-181 (Fig. 3). For this reason, we started with the $\alpha 3$-195-$\alpha 2$ subunit, in which the first 195 residues are derived from $\alpha 3$ and the remainder are derived from $\alpha 2$. We then changed the residue at position 198 from proline (as in $\alpha 2$) to glutamine (as in $\alpha 3$), to construct $\alpha 3$-195-$\alpha 2$,P198Q. Merely replacing the first 195 residues of $\alpha 2$ with $\alpha 3$ sequence was not enough to confer NBT sensitivity (Fig. 3A and 4), but the additional change of the residue at position 198 from proline to glutamine resulted in partial sensitivity to NBT blockade (51.8 ± 9.5% inhibition by 100 nM NBT). This result demonstrates the importance of Gln-198 in determining the NBT sensitivity of receptors formed by the $\alpha 3$ subunit.

Mapping of regions of α subunits involved in agonist sensitivity. We used the same set of chimeric subunits described above to identify regions of α subunits that are important in determining agonist sensitivity. As a measure of agonist sensitivity we determined the ratio of the responses to equal concentrations of nicotine and ACh. We previously showed (9) that $\alpha 2\beta 2$ is more sensitive to nicotine than to ACh and that $\alpha 3\beta 2$ is less sensitive to nicotine than to ACh over a range of concentrations below the EC_{50} for ACh for each receptor. In this study, we chose a concentration of 10 μM, which falls below the EC_{50} for ACh for both $\alpha 2\beta 2$ and $\alpha 3\beta 2$. For receptors formed with chimeric subunits, a concentration that fell below the EC_{50} for ACh for each receptor was also used. In most cases this was also 10 μM (see below). The wild-type $\alpha 2\beta 2$ receptor, which is much more sensitive to nicotine than to ACh, had a nicotine/ACh ratio of 5.65 ± 0.32. The wild-type $\alpha 3\beta 2$ receptor, which is much less sensitive to nicotine than to ACh, had a nicotine/ACh ratio of 0.17 ± 0.06.

Fig. 5A shows the agonist sensitivity of receptors formed by each of the series of chimeric subunits consisting of an amino-terminal portion of $\alpha 2$ followed by a carboxyl-terminal portion of $\alpha 3$. The nicotine/ACh ratio shifted from $\alpha 3$-like to $\alpha 2$-like in two discrete steps. Replacement of the first 84 amino acid residues of $\alpha 3$ with those of $\alpha 2$ ($\alpha 2$-84-$\alpha 3$) shifted agonist sensitivity from the nicotine/ACh ratio of wild-type $\alpha 3$ (0.17 ± 0.06) to a value intermediate between those of $\alpha 3$ and $\alpha 2$ (0.94 ± 0.19). This suggests that sequence segment 1-84 is important in determining agonist sensitivity. Replacement of $\alpha 3$ sequence with $\alpha 2$ sequence up to position 121, 181, or 195 caused no further change in the nicotine/ACh ratio. The second shift occurred only upon replacement of the first 215 residues of $\alpha 3$

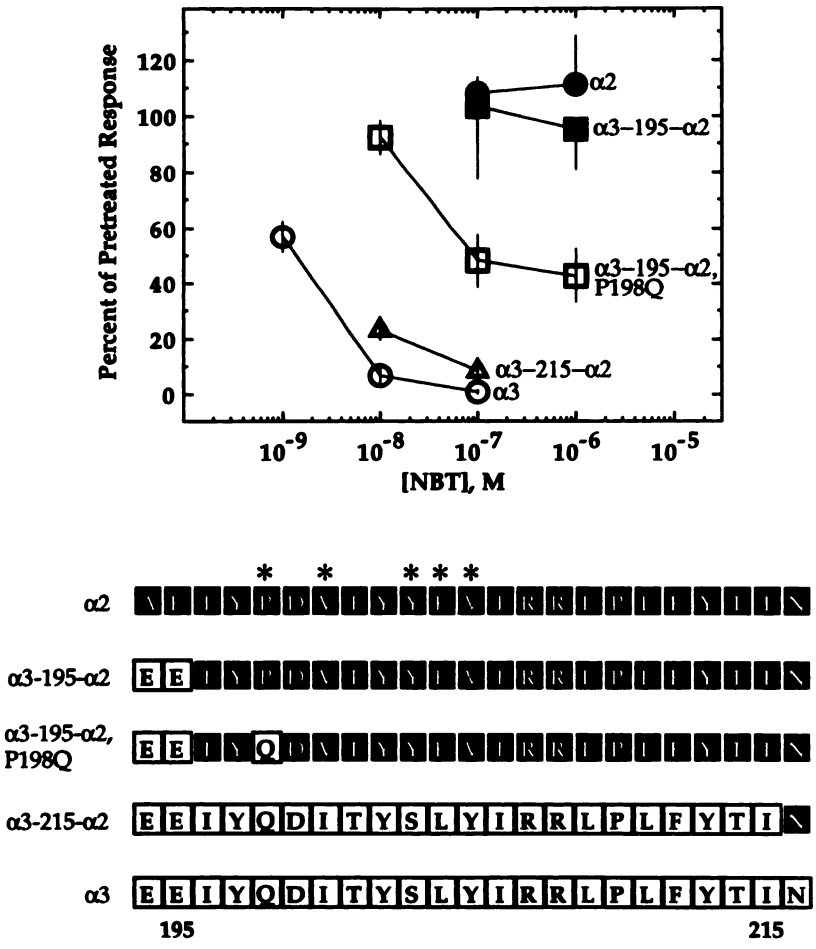

Fig. 4. Gln-198 of $\alpha 3$ is important for NBT sensitivity. *Upper*, ACh-induced current responses of oocytes expressing $\alpha 2$ (●), $\alpha 3$-195-$\alpha 2$ (■), $\alpha 3195$-$\alpha 2$,P198Q (□), $\alpha 3$-215-$\alpha 2$ (△), or $\alpha 3$ (○), in combination with $\beta 2$, were measured before and after incubation with various concentrations of NBT for 30 min. The post-treatment response is presented as a percentage of the pretreatment response (mean ± standard deviation of three to five occytes). *Lower*, the amino acid sequence between positions 195 and 215 is shown for each subunit. *Black boxes*, sequence derived from $\alpha 2$; *white boxes*, sequence derived from $\alpha 3$. *,Amino acid residues within this region that differ between $\alpha 2$ and $\alpha 3$. The mean percentage of pretreated response for $\alpha 3$-195-$\alpha 2$,P198Q is significantly different from $\alpha 2$ ($p < 0.001$) and $\alpha 3$ ($p < 0.01$) at 100 nM NBT, from $\alpha 2$ ($p < 0.01$) at 1 μM NBT, and from $\alpha 3$ ($p < 0.001$) at 10 nM NBT.

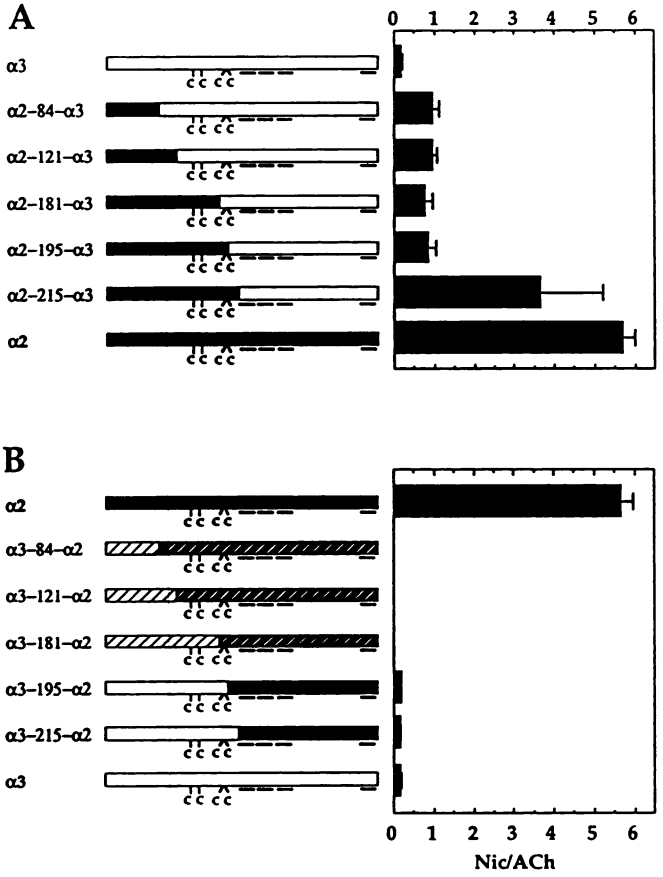

Fig. 5. Amino acids involved in agonist sensitivity are located within sequence segments 1-84 and 195-215. *Left*, schematic representations of the chimeric subunits. *Black*, sequence derived from α2; *white*, sequence derived from α3. *Right*, ratio of the responses to nicotine (*Nic*) and to ACh of oocytes expressing receptors formed by each chimeric subunit in combination with β2 (mean ± standard deviation of three to five oocytes). Significantly different from α3 are α2-84-α3 ($p < 0.001$), α2-121-α3 ($p < 0.001$), α2-181-α3 ($p < 0.01$), α2-195-α3 ($p < 0.01$), α2-215-α3 ($p < 0.01$), and α2 ($p < 0.001$). Significantly different from α2 are α2-84-α3 ($p < 0.001$), α2-121-α3 ($p < 0.001$), α2-181-α3 ($p < 0.001$), α2-195-α3 ($p < 0.001$), α2-215-α3 ($p < 0.05$), α3-195-α2 ($p < 0.001$), and α3-215-α2 ($p < 0.001$). A, Agonist sensitivity of receptors formed (in combination with β2) by each of a series of chimeric subunits in which increasingly large portions of the amino-terminal sequence of α3 were replaced by the corresponding portions of α2. B, Agonist sensitivity of receptors formed (in combination with β2) by each of a series of chimeric subunits in which increasingly large portions of the amino-terminal sequence of α2 were replaced by the corresponding portions of α3. *Hatch marks* on chimeric subunits α3-84-α2, α3-121-α2, and α3-181-α2 are to indicate that injection of RNA encoding each of these subunits in combination with RNA encoding β2 does not result in the expression of functional receptors.

with α2 sequence. This chimeric subunit (α2-215-α3) formed receptors with a nicotine/ACh ratio of 3.65 ± 1.58, similar to that of wild-type α2β2. This result suggests that amino acids involved in determining agonist sensitivity are also located between position 195 and 215.

Fig. 5B shows the agonist sensitivity of chimeric subunits with α3 sequence at the amino terminus. Again, we were unable to demonstrate functional expression of chimeric subunits in which α2 sequence was replaced with α3 sequence up to positions 84, 121, or 181 (see Experimental Procedures). Replacement of the first 195 amino acid residues of α2 with α3 sequence (α3-195-α2) resulted in receptors with an agonist sensitivity similar to that of receptors formed by wild-type α3. Replacement of α2 sequence with α3 sequence up to position 215 (α3-215-α2) also resulted in receptors with a wild-type α3 agonist sensitivity. Although the nonfunctional chimeric subunits limited the resolution of experiments involving this set of chimeric subunits, we can conclude that the replacement of the first 195 residues of α2 with α3 sequence results in a change in agonist sensitivity.

Importance of the amino acid residue at position 198 for agonist sensitivity. We used site-directed mutagenesis to examine the role of individual amino acid residues in determining agonist sensitivity. Because the identity of the amino acid at position 198 (glutamine in α3 and proline in α2) was shown in Fig. 4 to be important for NBT sensitivity, we were particularly interested in whether the amino acid at this position might also play a role in determining agonist sensitivity. We also examined the role of the asparagine at position 191 of α3 (aspartate in α2) and the glutamate at position 194 of α3 (alanine in α2).

Fig. 6 shows the agonist sensitivity of receptors formed by mutant and wild-type α subunits. The wild-type α2 subunit (Fig. 6, *top*) forms receptors that are much more sensitive to nicotine than to ACh (nicotine/ACh ratio = 5.65 ± 0.32). Changing the α2 amino acid sequence at position 191 from aspartate to asparagine, at position 194 from alanine to glutamate, and at position 198 from proline to glutamine (α2,D191N,A194E,P198Q) had a profound effect on the nicotine/ACh ratio of the resulting receptor (0.64 ± 0.10). Receptors formed by the α2 subunit in which only position 198 had been changed (α2,P198Q) had a nicotine/ACh ratio of 0.90 ± 0.05. In contrast, altering the residues at positions 191 and 194 (α2,D191N,A194E) had much less effect on agonist sensitivity (nicotine/ACh ratio = 2.96 ± 0.25). Thus, of the three amino acid changes, it is the change at position 198 that is primarily responsible for the change in agonist sensitivity. When the α3 amino acid sequence was changed at position 191 from asparagine to aspartate, at position 194 from glutamate to alanine, and at position 198 from glutamine to proline (α3,N191D,E194A,Q198P), the resulting subunit formed receptors with a nicotine/ACh ratio (0.39 ± 0.07) only slightly (but significantly, $p < 0.01$) different from that of receptors formed by native α3. Thus, the residue at position 198 may play a role in determining the agonist sensitivity of α2β2, but changing from proline to glutamine at this position is not sufficient to confer α2β2 agonist sensitivity on the α3β2 receptor.

Increased sensitivity to ACh displayed by receptors composed of chimeric subunits formed at position 84, 121, or 181. The ACh sensitivity of each of the receptors in this study was estimated by construction of ACh dose-response curves. Given the rapid desensitization of nAChRs that occurs at high agonist concentrations and the difficulty of applying agonist rapidly to cells the size of *Xenopus* oocytes, EC_{50} values derived from these dose-response curves must be considered approximate (see Experimental Procedures). Most of the chimeric and mutant subunits formed receptors that displayed EC_{50} values for ACh that were similar to those obtained for receptors formed by wild-type α2 or α3 (approximately 100 μM and 20 μM, respectively). Receptors formed by these subunits, as well those formed by α2 or α3, were studied using 10 μM ACh and 10 μM nicotine to determine the nicotine/ACh ratio and using 1 μM ACh to determine the sensitivity to NBT.

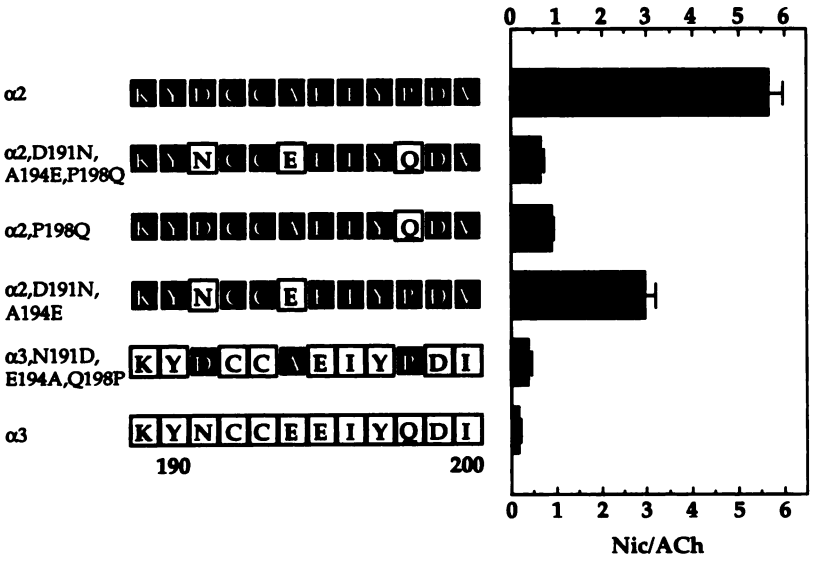

Fig. 6. The amino acid residue at position 198 is important for agonist sensitivity. *Left*, portion of amino acid sequence where mutations have been made, for each subunit. *Black boxes*, sequence derived from α2; *white boxes*, sequence derived from α3. *Right*, ratio of the responses to nicotine (*Nic*) and to ACh of oocytes expressing receptors formed by each mutant subunit in combination with β2 (mean ± standard deviation of three to five oocytes). Significantly different from α2 are α2,D191N,A194E,P198Q ($p < 0.001$), α2,P198Q ($p < 0.001$), α2,D191N,A194E ($p < 0.001$), and α3,N191D,E194A,Q198P ($p < 0.001$). Significantly different from α3 are α2,D191N,A194E,P198Q ($p < 0.001$), α2,P198Q ($p < 0.001$), α2,D191N, A194E ($p < 0.001$), and α3,N191D,E194A,Q198P ($p < 0.01$).

Chimeric subunits in which the first 84, 121, or 181 amino acid residues of α3 were replaced with α2 sequence formed receptors that were much more sensitive to ACh than were receptors formed by wild-type α2 or α3. Dose-response curves for receptors formed by α2 and α3, as well as chimeric subunits joined at positions 84 and 181, are shown in Fig. 7. The EC_{50} values for ACh of α2β2 and α3β2 were approximately 100 μM and 20 μM, respectively. In contrast, replacement of the first 84 or the first 181 residues of α3 with α2 sequence resulted in subunits that formed receptors with EC_{50} values for ACh of approximately 1.5 μM and 80 nM, respectively. Also, replacement of the first 121 residues of α3 with α2 sequence resulted in a subunit that formed receptors with an EC_{50} for ACh of approximately 700 nM (data not shown). This leftward shift in the dose-response curve could be the result of a change in the affinity of the binding site for agonist, in the efficacy of agonist in activating the receptor, or in the channel kinetic properties of the receptor. Although we do not currently understand the reason for this shift in ACh sensitivity, we have attempted to minimize the effect of this shift on our experiments by lowering the agonist concentrations that were used to determine the nicotine/ACh ratio of these receptors (300 nM for α2-84-α3β2, 100 nM for α2-121-α3β2, and 30 nM for α2-181-α3β2). Interestingly, the chimeric subunits that formed receptors highly sensitive to ACh are the mirror images of the nonfunctional chimeric subunits, in which α2 sequence was replaced by the first 84, 121, or 181 amino acid residues of α3. The other nine chimeric and mutant subunits formed receptors that displayed EC_{50} values for ACh that were similar to the EC_{50} values for ACh of receptors formed by wild-type α2 or α3. The EC_{50} values of receptors formed by α2-195-α3 and α2-215-α3 were similar to that of receptors formed by α2 (approximately 100 μM), and the EC_{50} values for ACh of receptors formed by α3-195-α2 and α3-215-α2 were similar to that of receptors formed by α3 (approximately 20 μM). The EC_{50} values of receptors formed by each of the mutant subunits (α2,D191N,A194E,P198Q, α2,P198Q, α2,D191N,A194E, α3,N191D,E194A,Q198P, and α3-195-α2,P198Q) were all between 20 μM and 100 μM.

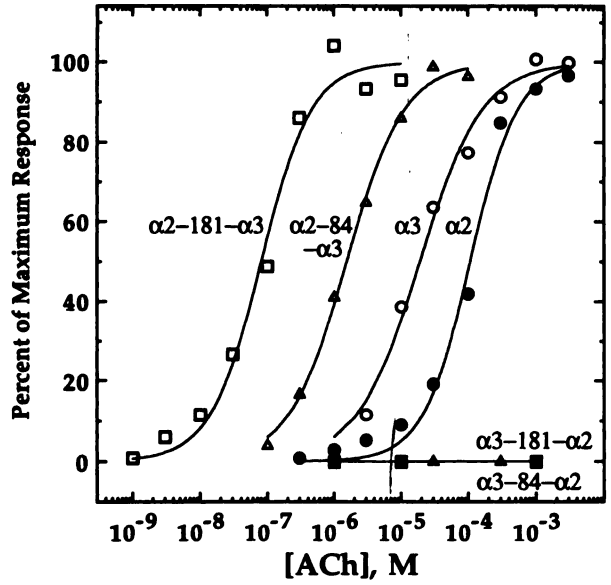

Fig. 7. Receptors formed by α2-84-α3 or α2-181-α3 are more sensitive to ACh than are receptors formed by α2 or α3. ACh dose-response curves were constructed for oocytes expressing α2β2 (●), α3β2 (○), α2-84-α3β2 (△), α3-84-α2β2 (▲), α2-181-α3β2 (□), or α3-181-α2β2 (■). Oocytes injected with RNA encoding α3-84-α2 or α3-181-α2, in combination with RNA encoding β2, did not respond to any concentration of ACh. Each *point* represents the mean of responses from two to six separate oocytes.

Discussion

Neuronal nAChRs formed by the α2 or α3 subunits differ dramatically in their sensitivity to nicotinic agonists and antagonists. By constructing and pharmacologically analyzing chimeric subunits consisting of portions of these two α subunits, we have mapped the location of sequence segments that confer differences in agonist and antagonist sensitivity. We have identified the region from the amino terminus to position 84 as important in determining sensitivity to the agonists ACh and nicotine but having little importance in determining sensitivity to the antagonist NBT. The regions from position 84 to 121 and from position 121 to 181 contain amino acid residues important in determining NBT sensitivity. The sequence segment from position 195 to 215 is important for both agonist and antagonist sensitivity. Within this region, we have identified the amino acid residue at position 198 (glutamine in α3

and proline in α2) as being important in determining the sensitivity of neuronal nAChRs to both agonists and antagonists. Interestingly, this residue is a proline in α4, which forms receptors with β2 that are only weakly sensitive to NBT (8) and are highly sensitive to nicotine (24).

Within the sequence segments that we have identified are several highly conserved amino acid residues that have been located at the ligand binding site of the *Torpedo* nAChR. The regions from position 84 to 121 and from position 121 to 181 are important for sensitivity to NBT (Fig. 3A) and contain the residues Tyr-93 and Trp-149, respectively. These two residues of the *Torpedo* α subunit are photolabeled by the competitive antagonist [^{3}H]DDF (14, 16). The region from position 195 to 215 is important in determining both agonist and antagonist sensitivity and contains a tyrosine residue at a position (197 in α3) analogous to Tyr-198 of the *Torpedo* α subunit. Tyr-198 of the *Torpedo* α subunit is photolabeled by [^{3}H]nicotine (17). Because each of these residues is common to both α2 and α3, they cannot be responsible for pharmacological differences between receptors formed by these two subunits. Other residues within the identified sequence segments must then be responsible for the dramatic differences in the pharmacological properties of receptors formed by α2 and α3. We have identified such an amino acid residue directly adjacent to Tyr-197 of α3. This residue, Gln-198 of α3 (proline in α2), is involved in determining the sensitivity of neuronal nAChRs to both agonists and antagonists. This residue might be an actual participant in binding agonists and antagonists. The difference in the ability of glutamine and proline to form hydrogen bonds could be important in determining pharmacological specificity. Another possibility is that the greater degree of conformational freedom of the glutamine residue, compared with the proline residue, results in a change in the overall shape of the binding site. These ideas can be tested by more extensive mutagenesis experiments that introduce different residues into this position.

A recent study by McLane *et al.* (25) attempted to localize sequence segments of α3 that form the binding site for NBT. A series of overlapping peptides corresponding to the entire α3 sequence were synthesized and tested for the ability to bind ^{125}I-NBT. The peptides α3:51–70 and α3:1–18 were shown to specifically bind ^{125}I-NBT in a solid-phase assay, with K_d values of approximately 300 nM and 500 nM, respectively. These peptides were also shown to inhibit the binding of ^{125}I-NBT to PC12 cells, a cell line possessing NBT binding sites and expressing the α3 subunit. A peptide corresponding to the sequence of α2 analogous to α3:51–70 did not bind NBT. The portions of α3 sequence from positions 1 to 18 and from positions 51 to 70 were therefore hypothesized to form an important part of the NBT binding site on α3. Our experiments involving construction of chimeric subunits offered a way to directly test this hypothesis. We found, however, that the portion of α3 from positions 1 to 84, which includes both α3:1–18 and α3:51–70, was of little importance for the NBT sensitivity of the receptor. Replacement of the first 84 amino acid residues of α3 with α2 sequence resulted in a subunit that formed receptors nearly as sensitive to NBT blockade as were receptors formed by wild-type α3 (Fig. 3A). McLane *et al.* (25) also showed that the peptides α3:180–199 and α3:183–201, although not able to bind ^{125}I-NBT in a solid-phase assay, were able to partially inhibit the binding of ^{125}I-NBT to PC12 cells. A region of α3 that we have identified as being important for NBT sensitivity, segment 195-215, overlaps with these two peptides. Furthermore, amino acid residue Gln-198 lies within this region of overlap. Thus, although studies using peptides may identify sequence segments important for ligand binding (α3:180–199 and α3:183–201), the relative affinities of different peptides for ligands may not be relevant to the role those sequences play in the intact molecule.

We have chosen to use simple pairwise combinations of α and β subunits in our study of the ligand binding site. However, the subunit composition of neuronal nAChRs *in vivo* is currently uncertain. Expression studies in *Xenopus* oocytes suggest that neuronal nAChRs are formed by simple subunit combinations, for example, one kind of α subunit and one kind of β subunit. Immunoprecipitation experiments suggest that the high affinity nicotine receptor in rat brain is just such a simple combination, α4 and β2 (24). However, recent work by Vernallis *et al.* (26) suggests that some neuronal nAChRs may have a more complex subunit composition. Receptors immunoprecipitated from chick ciliary ganglion were shown to contain the α3, α5, and β4 subunits.

The binding sites on nAChRs have long been thought to reside solely on the α subunits, but recent work suggests that the binding sites on muscle-type nAChRs are located at the interfaces between the α and γ subunits and the α and δ subunits (17–20). The binding sites of the neuronal nAChRs may be formed in a similar manner, because both α and β subunits are involved in determining the pharmacological properties of these receptors (6, 9). We have identified sequence segments that are responsible for the contribution of α subunits to the pharmacological properties of neuronal nAChRs. A similar approach has been used to identify amino acid residues responsible for the contribution of the β subunits. The presence of either β2 or β4 has a profound effect on the responsiveness of receptors to the agonist cytisine (9). By generating chimeric β subunits, Figl *et al.* (27) have shown that this difference is determined primarily by residues 108 (phenylalanine in β2 and valine in β4) and 110 (serine in β2 and threonine in β4). The β subunits also affect antagonist sensitivity. Although the α3β2 subunit combination is sensitive to blockade by NBT, the α3β4 subunit combination is insensitive to NBT (6). Amino acid residues responsible for this difference have been mapped to the first 133 residues of the β subunits.[3]

The binding site for the quaternary ammonium of ACh has traditionally been thought to consist of negatively charged amino acids. In fact, negatively charged residues on the *Torpedo* δ subunit have been identified as forming part of the ACh binding site on *Torpedo* nAChR (20). A number of recent studies suggest that aromatic residues may also participate in forming the quaternary ammonium binding site. Work with completely synthetic structures, which bind ACh and other quaternary ammonium compounds with reasonably high affinity, suggests that quaternary nitrogens can interact with the π electrons of aromatic groups (28). Also, in X-ray diffraction studies of antiphosphocholine antibodies, both negatively charged and aromatic residues were found to form a binding pocket around the charged nitrogen (29). The recent solution of the atomic structure of AChE has provided a detailed look at the structure of an ACh binding site (30). The active site of this enzyme lies at the bottom of a deep narrow gorge lined with several negatively charged residues as well as 14 aromatic residues. These aromatic amino acids appear to form a sub-

stantial part of the "anionic" subsite of AChE. The ACh binding site of nAChRs could have general structural similarity to the active site gorge of AChE. Consistent with this idea is the labeling of aromatic residues by [^{3}H]DDF, [^{3}H]lophotoxin, and [^{3}H]nicotine (14–17). These residues might form the walls of a binding site gorge on the nAChR. Highly conserved among nAChR subunits, these aromatic residues may form portions of the binding site common to all nAChRs but clearly cannot be responsible for the diversity seen among these receptors. This diversity might be conferred by differences in some of the residues that participate in the binding of ligand. Alternatively, this diversity might be due to differences in residues that, without actually participating in binding, affect the overall conformation of the binding site. The residue that we have identified, Gln/Pro-198, could play either of these roles.

Our use of chimeric and mutant receptor subunits has enabled us to map the location of amino acid residues responsible for pharmacological diversity among neuronal nAChRs. We have identified several sequence segments (positions 84-121, 121-181, and 195-215) of the α3 subunit that are involved in determining the sensitivity of neuronal nAChRs to NBT blockade. Sequence segments 1-84 and 195-215 were found to be important in determining the sensitivity of neuronal nAChRs to ACh and nicotine. We identified the amino acid residue at position 198 (glutamine in α3 and proline in α2) as being important in determining both the antagonist sensitivity and the agonist sensitivity of these receptors. Continued use of this chimeric/mutant technique will allow the identification of the complete set of amino acid residues that determine the pharmacological specificity of neuronal nAChRs.

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