The Effects of β3 Subunit Incorporation on the Pharmacology and Single Channel Properties of Oocyte-expressed Human α3β4 Neuronal Nicotinic Receptors*

James P. Boorman‡, Marco Beato§, Paul J. Groot-Kormelink§, Steven D. Broadbent§, and Lucia G. Sivilotti¶

Received for publication, November 18, 2002, and in revised form, July 24, 2003
Published, JBC Papers in Press, August 11, 2003, DOI 10.1074/jbc.M211719200

The Journal of Biological Chemistry Vol. 278, No. 45, Issue of November 7, pp. 44033–44040, 2003

© 2003 by The American Society for Biochemistry and Molecular Biology, Inc.

We compared the main properties of human recombinant α3β3 neuronal nicotinic receptors with those of α3β4 receptors, expressed in Xenopus oocytes. β3 incorporation decreased the channel mean open time (from 5.61 to 1.14 ms, after approximate correction for missed gaps) and burst length. There was also an increase in single channel slope conductance from 28.8 picosiemens (α3β4) to 46.7 picosiemens (α3β4β3; in low divalent external solution). On the other hand, the calcium permeability ability (determined by a reversal potential method in 3693; Fax: 44-20-7679-3693; Tel: 44-20-7679-7298; E-mail: l.sivilotti@ucl.ac.uk.

Among neuronal nicotinic subunits, β3 was long considered an “orphan” as it does not form functional recombinant receptors if expressed as a classical heteromeric combination, i.e., together with either an α or a β subunit. Because of the sequence similarity between β3 and α5 we tested the hypothesis that, just like α5, β3 would form functional receptors only if co-expressed together with both an α and a β subunit. By a reporter mutation strategy, we showed that β3 is indeed incorporated into functional recombinant α3β4β3 receptors (1). In α3β4β3 receptors, β3 is present as a single copy, which replaces one of the β subunits (versus two copies each for the α3 and β4 subunits (2); note that β3 coassembles with other β subunits in rat native cerebellar nAChRs (3).

The question now arises of whether the β3 subunit can change the properties of neuronal nicotinic receptors. There are several reasons for investigating this problem. First of all it is important to establish whether any such change introduced by the presence of β3 affects the role that these receptors may have in physiological processes in the central nervous system or in the pharmacology of tobacco addiction. Furthermore, if β3-containing receptors have distinctive biophysical or pharmacological properties, such receptors can in principle be recognized in native tissue by functional assays. Finally, clear changes in the receptor pharmacology and particularly in the binding affinity of competitive antagonists would be a strong indication that the binding sites of the receptor have changed and that β3 directly forms one of the two interface binding sites.

The shorter openings and bursts together with the increased single-channel conductance observed with the co-expression of β3 confirm that β3 is incorporated into the receptor complex. The single-channel conductance change and the stoichiometry of the receptor (2) suggest that β3 takes the place of a classical β subunit. The calcium permeability of the receptor and its sensitivity to a range of agonists and antagonists were unchanged apart from a decrease in the relative potency of the agonist lobeline. These results suggest that β3 either does not participate in the formation of the agonist binding site or that the sequences of β3 and β4 in the relevant domains are too similar to allow them to be differentiated.

The extent and nature of changes introduced into the nAChR by the presence of β3 were not sufficient to provide tools for the identification of such receptors in native tissue. Nevertheless, the shorter burst length of these receptors implies a faster decay of any synaptic current that these channels may mediate and suggests a functional role for the β3 subunit in the diverse family of neuronal nicotinic receptor subunits.

Among neuronal nicotinic subunits, β3 was long considered an “orphan” as it does not form functional recombinant receptors if expressed as a classical heteromeric combination, i.e.

* This work was supported by Wellcome Trust Project Grants 055524 and 064652, Medical Research Council Cooperative Grant GB819400 Ph.D. studentship (to S. D. B.), and a School of Pharmacy Millennium Studentship (to J. P. B.).

‡ Current address: Dept. of Biology, University College London, London WC1E 6BT, UK.

§ Current address: Dept. of Pharmacology, University College London, London WC1E 6BT, UK.

¶ To whom correspondence should be addressed. Tel.: 44-20-7679-3693; Fax: 44-20-7679-7298; E-mail: l.sivilotti@ucl.ac.uk.

The abbreviations used are: nAChR, nicotinic acetylcholine receptor; ACh, acetylcholine; DMPP, 1,1-dimethyl-4-phenylpiperazinium.
EXPERIMENTAL PROCEDURES

Expression of Nicotinic Subunits cRNA in the Xenopus oocyte—cDNAs for the human α3, β3, and β4 (GenBank accession numbers Y098417, Y098418, and Y098417, respectively) coding sequences and an added Kozak consensus sequence (GCCACC) immediately upstream of the start codon (4) were subcloned into the PSB64GL vector, which contains 5′- and 3′- untranslated Xenopus β-globin regions (5). All cDNAs were linearized immediately downstream of the 3′-untranslated β-globin sequence, and cRNA was transcribed using the SP6 Message Machine Kit (Ambion). The quality and quantity of cRNAs were checked by gel electrophoresis and comparison with RNA concentration and size markers.

Female Xenopus laevis frogs were anesthetized by immersion in neutralized ethyl m-amino-benzoate solution (tricaine, methane sulfonate salt; 0.2% solution weight/volume) and killed by decapitation and destruction of the brain and spinal cord (in accordance to Home Office guidelines) before removal of the ovarian lobes. Clumps of stage V-VI oocytes were dissected in a sterile modified Barth’s solution with a composition of 88 mM NaCl, 1 mM KCI, 0.82 mM MgCl2, 0.77 mM CaCl2, 2.4 mM NaHCO3, 15 mM Tris-HCl in high performance liquid chromatography-grade water with 50 units ml−1 penicillin and 50 μg ml−1 streptomycin (Invitrogen), pH 7.4, adjusted with NaOH. The dissected oocytes were treated with collagenase (type IA, Sigma; 65 min at 18 °C, 245 collagen digestion units ml−1 in Barth’s solution, 10–12 oocytes/ml), rinsed, stored at 4 °C overnight, and manually defolliculated the following day before cRNA injection (46 nl/oocyte). The oocytes were incubated for ~60 h at 18 °C in Barth’s solution containing 5% heat-inactivated horse serum (Invitrogen) (6) and then stored at 4 °C. Experiments were carried out at a room temperature of 18–20 °C between 2.5 and 14 days from injection.

cRNA was injected at a ratio of 1:1 in order to express α3β4 receptors and at a ratio of 1:1:20 (α3:β3:4) in order to express α3β4β3 receptors in conditions that minimized the presence of pair α3β4 receptors. We have previously found that with this ratio the proportion of current through α3β4 receptors is too small to be detected by fitting dose-response curves, since it is 39% when the ratio for α3:β3:4 is 1:1:1 (11). The total amount of cRNA to be injected (46 nl of RNase-free water) for each combination was determined empirically, with the aim of achieving an optimal signal to noise ratio in the different experiments. Given that most of the experiments described here were carried out at low agonist concentrations, the level of expression we tried to achieve was higher than that needed for full agonist dose-response curves. The average quantity of cRNA injected in each oocyte was 20 and 30 ng for α3β4 and α3β4β3, respectively.

Single Channel Recording—Recordings were obtained in the cell-attached configuration from oocytes that had been stripped of their vitelline membrane after incubation in hyperosmotic solution (350 mOsmol/kg H2O) with 20 mM KCl, 1 mM MgCl2, 10 mM HEPES, pH 7.4, with NaOH. Electrodes were pulled from thick-walled borosilicate glass (GC150F, Warner Instruments) coated with Sylgard® 184 (Dow Corning) and fire-polished to have a final resistance of 12–15 megaohms when filled with low divalent external solution (150 mM NaCl, 2.5 mM KCl, 0.5 mM MgCl2, 0.5 mM CaCl2, 10 mM HEPES, 0.5 μM atropine, pH 7.2, with NaOH which contained 1 μM ACh as agonist. No channel openings other than those attributable to stretch channels were observed in the absence of ACh in the external solution (7). Oocytes were immersed in high potassium solution (100 mM NaCl, 10 mM HEPES, 10 mM EGTA, pH 7.2, with KOH) to drive the resting membrane potential of the oocyte to a value consistent and close to 0. Recordings were filtered at 3 kHz (8-pole Bessel filter) and digitized at 30 kHz. The recordings used for amplitude analysis were idealized by time-course fitting; fitted amplitude distributions were fitted with gaussian curves (SCAN and EKDIST, courtesy of D. Colquhoun, UCL; www.ucl.ac.uk/Pharmacology/dcpr95.html) to give a mean value for the amplitudes of the openings at 150-, 125-, 100-, 75-, and 50-mV holding potentials; the mean values from each patch (4 and 3 patches for α3β4 and α3β4β3, respectively) were pooled for the different holding potentials to give the points in Fig. 1C, which were fitted with a straight line to obtain slope conductance values (CVFIT, courtesy of D. Colquhoun, UCL). Errors were calculated from the residuals.

Recordings at a holding potential of 100 mV were used for obtaining dwell time distributions and idealized by a threshold crossing method with a resolution of 80 μs. Open period and shut time distributions were fitted with a mixture of exponential components (PClamp, Axon Instruments). An approximate correction of the mean open time for missed bursts was carried out as previously detailed (8).

Bursts were defined as groups of openings separated by shut times which were shorter than a particular value, tSHUT. We chose the tSHUT value by inspecting shut time distributions and choosing the one for which the time constants of their components across patches, since the time constants of gaps between bursts are expected to vary across patches because they depend on both the agonist concentration and the number of channels in a patch. The tSHUT value was on average 168 μs for α3β4 and 5.6 μs for α3β4β3.

Partial (two-point) concentration-response curves were obtained at low agonist concentrations first in control solution (modified Ringer solution; this was present both in the Ringer and in the agonist solutions). The antagonist experiments were carried out only after checking that this standard concentration gave reproducible responses. The average changes in the response to this ACh standard concentration observed by the end of the experiment for the different combinations ranged from −58 to +60%. All the data are compensated for the response rundown. However, applying this compensation did not affect the conclusions of our work, and the original data (without compensation) were similar (data not shown). For details specific to each type of experiment, see below.

Agonist Potency Ratios—These were obtained following a previously described protocol (10). The aim of this was to obtain partial (2- or 3-point) dose-response curves at the lowest agonist concentrations that gave an acceptable signal to noise ratio. Care was taken to match the response size for all the agonists tested in a given experiment. These conditions were chosen to reduce the contributions of desensitization and agonist self block and essentially ensured that the slope of the dose-response curves was similar for all the agonists (see the examples in Fig. 3 and 4). Given the large number of agonists tested, only a subset of agonists could normally be tested in one cell, but ACh was tested in every experiment and was used as a standard. All curves obtained in each experiment were fitted simultaneously by least squares (CVFITT, courtesy of David Colquhoun, UCL; see www.ucl.ac.uk/Pharmacology/dcpr95.html) with power functions constrained to be parallel (these are the equivalent of fitting with a Hill equation in which the maximum is constrained to be very large with respect to the measured responses). In these simultaneous fits the program estimated the distances between the dose-response curves of the different agonists and that of the standard ACh. Such distances were obtained as concentration ratios with respect to ACh. The potency ratios shown in Table II are the reciprocal of the concentration ratios; errors and confidence intervals for potency ratios were calculated by Fidler’s theorem (11). When the constraint of parallelism was justified, data were fitted without it to obtain estimates of the Hill slope for each agonist (see Table II). The maximum agonist concentrations used were 0.03 μM for epibatidine, 1.5 μM for lobeline, 5 μM for cytisine, 10 μM for 1,1-dimethyl-4-piperidyl-piperazinium iodide (DMPP), 20 μM for ACh and nicotine, and 200 μM for atropine. Note that 20 μM ACh processes less than 5% of its maximum response for both combinations tested.

Antagonist Experiments—The antagonist experiments were carried out to confirm the competitive nature of the antagonist action and to estimate the antagonist dissociation constant by the Schild method. Partial (two-point) concentration-response curves were obtained at low agonist concentrations first in control solution (modified Ringer + nM calcium) and then in the presence of an appropriate antagonist concentration; this was present both in the Ringer and in the agonist solutions. Oocytes were incubated with the antagonist for 25 min before responses to ACh were again tested. Only one antagonist concentration was tested
in each oocyte in order to minimize the distortion by response rundown (which cannot be quantified in the presence of the antagonist). Only data from oocytes in which the agonist response recovered after antagonist treatment to at least 60% of control were included in the analysis. In a manner similar to that employed for the agonist potency ratios, the dose ratio \( r \) produced by the antagonist was measured for each experiment by fitting to the partial concentration-response curves power functions constrained to be parallel. Unconstrained fits were also performed; there was no significant difference between the slope of the concentration response in control conditions (range 1.59–1.71) and in 0.2–1 \( \mu \)M trimetaphan (range 1.37–1.73, paired \( t \) test).

To check that the data were adequately fitted by the Schödl equation, data were plotted as a Schödl plot and fitted by a power function, \( r = \alpha[B]^n \), where \( [B] \) is the concentration of antagonist, and \( \alpha \) is a constant, in order to estimate the Schödl slope \( n \). Having found \( n \) to be close to 1 (see Table III), we refitted the data with the Schödl equation, \( r = \frac{1-[B]/K_B}{[B]/K_B} \), to obtain \( K_B \), the antagonist dissociation equilibrium constant.

**Calcium Permeability**—We measured the effect of different calcium concentrations on the reversal potential of the current induced by 2-s voltage ramps from concentrations on the reversal potential of the current induced by 2-s voltage ramps from 100 mV holding potential. Such ramps were applied in the depolarizing direction and in the hyperpolarizing direction both in control conditions (leak) and in the presence of ACh (test) once the agonist current was steady. Current-voltage curves were averaged (\( n = 4 \)), and leak was subtracted using PClamp (Axon Instruments). Responses were sampled at 2 kHz (Axon Digidata 1320), filtered at 500 Hz (8-pole Bessel filter), and stored on disk for later analysis.

The contribution of the cell endogenous calcium-dependent chloride conductance (which would be activated by calcium entry through nicotinic channels) was minimized by preincubating the oocytes in chloride-free low calcium Ringer solution for 24 h (115 mM NaOH, 35 mM sucrose, 2.5 mM potassium gluconate, 10 mM HEPES, 1.8 mM Ca(OH)\(_2\), 0.5 mM atropine bromide, pH 7.2, with methanesulfonic acid). Experiments were also carried out in chloride-free modified Ringer’s solution. The composition of the low calcium Ringer was 1.8 mM Ca(OH)\(_2\), 35 mM sucrose, 115 mM NaOH, 2.5 mM potassium gluconate, 10 mM HEPES, 0.5 mM atropine bromide, pH 7.2, with methanesulfonic acid; the high calcium Ringer contained 18 mM Ca(OH)\(_2\) and no sucrose (osmolarity was 263 and 261 mosmol/liter, respectively). All recordings were carried out with electrodes filled with 2.5 \( \mu \)M potassium acetate and 10 mM KCl using a 3 \( \times \) KCl agar bridge as a reference electrode.

**Materials**—The following compounds were purchased from Sigma: acetylcholine chloride, atropine methyl bromide or sulfate, calcium acetate, carbamylcholine chloride (carbachol), (L)-epibatidine hydrochloride, (D)-cytisine, dihydro-\( \beta \)-erythroidine hydrobromide, DMPP, (L)-nicotine hydrochloride, (D)-nicotine hydrogen tartrate, potassium acetate, potassium gluconate, and tricaine methanesulfonate. Trimetaphan camsylate was from Cambridge Laboratories Ltd., Walsend, UK. All other chemicals were from BDH, Analar grade.

**RESULTS**

**Single Channel Properties**—As shown by the cell-attached records in Fig. 1, A and B, co-expression of the \( \beta 3 \) subunit had a profound effect on the properties of oocyte-expressed \( \alpha 3\beta 4 \) neuronal nicotinic receptors. The traces (obtained in the presence of 1 \( \mu \)M ACh at \(-100 \) mV) show that the openings of \( \beta 3 \)-containing receptors were larger and occurred in much shorter bursts (see also the expanded sweeps to the right of the continuous records).
The open point histograms in the same figure (scaled to unitary area) show that a single amplitude class accounted for the openings. Gaussian fits of the open point histogram for these two patches gave amplitude values of 3.13 ± 0.75 and 4.00 ± 0.69 pA for α3β4 and α3β4β3, respectively. Note the width of the open point histogram, which indicates a relatively large open channel noise, a well known phenomenon for neuronal nicotinic receptors be they native or recombinant (12, 13). The slope conductance of these channels (measured by fitting current-voltage plots in Fig. 1C) was 28.8 ± 0.66 picosiemens (4 patches) for the α3β4 combination and 46.7 ± 1.76 picosiemens for α3β4β3 receptors (3 patches).

The most pronounced effect of the presence of the β3 subunit in the receptor complex was on the kinetics of the channel. The continuous recordings of Fig. 1 show clearly that most of the openings of α3β4 channels occur in very prolonged bursts. These long bursts were completely absent from recordings from α3β4β3 channels.

Fig. 2 shows dwell time distributions for representative patches for the two subunit combinations (see Table I). β3 incorporation produced a consistent shift toward shorter apparent open periods (9.4 to 1.8 ms). Thus, the major component in the apparent open period distribution was 3.3 ms for β3-containing receptors (45% of all openings) and 17 ms for α3β4 (38% of all openings). Shutter time distributions were all fitted with a mixture of five exponential components; the shortest components (the first 4 for α3β4 and the first 2 for α3β4β3) were consistent from patch to patch (Table I) and were, therefore, classed as within burst (see “Experimental Procedures”). The faster shutter component is shorter in α3β4 patches, and the majority of events in this component will be below experimental resolution; this would result in the apparent lengthening of α3β4 openings. Given that it is likely that few openings are missed at our experimental resolution of 80 μs, we applied an approximate correction for missed gaps (8); this did not change the effect of β3 incorporation because corrected open times were 5.6 and 1.1 ms in α3β4 and α3β4β3, respectively (Table I).

Measurements of average burst length confirmed the substantial shortening that is so striking from visual inspection of the traces. Thus mean burst length was 270 and 5.6 ms for α3β4 and α3β4β3 receptors, respectively (Table I). As Fig. 1 shows, a potential complication is that there are signs of heterogeneity in α3β4 bursts. We therefore considered the possibility that the population of shorter bursts was similar to the α3β4β3 bursts and simply became predominant with β3 incorporation. To assess this hypothesis, we measured mean burst length in α3β4 patches after excluding long bursts (the threshold for exclusion was chosen by inspection and was 274 ms on average in the 10 patches analyzed); the average duration for the “shorter” bursts was 64 ± 14 ms (n = 10) and, therefore, still substantially longer than that of α3β4β3 bursts.

Agonist Potency—There were no obvious differences between α3β4 and α3β4β3 receptors in the time course of agonist responses (see Fig. 3A) or the rank order of potency for an extensive series of agonists (epibatidine > lobeline > cytisine, DMPP, nicotine > ACh > carbachol; Table II, Fig. 3B). This rank order of potency is very similar to that found for human or rat α3β4 receptors by Chavez-Noriega et al. (14) and Meyer et al. (15) by EC50 comparisons in oocytes or HEK293 cells.

Nevertheless, there was a marked decrease in the potency of lobeline with the incorporation of β3, from 23.0 ± 3.70 to 7.14 ± 1.1, relative to ACh for the α3β4 and α3β4β3 receptors, respectively; see the different distances between the partial dose-response curve to lobeline (leftmost in Fig. 4B, filled circles) and that to the standard, ACh (filled squares).

The Hill slopes for each agonist were similar on the two different combinations (see Figs. 3B and 4B, Table II, t test). A fairly wide range of Hill slope values is reported in the literature for the same α3β4 receptor from values comparable with those observed here (10) to values in excess of 1.9 (14). Note that the latter were observed in the presence of low (0.18 mm...
extracellular calcium rather than nominally zero calcium (present study and Ref. 10).

Antagonist Potency—Trimetaphan acts as a competitive antagonist on native ganglion nAChRs (16), which are likely to contain the α3 and β4 subunits. A measurement (by Schild analysis) of the dissociation constant of trimetaphan would be useful for receptor classification and binding site modeling (very few true equilibrium dissociation constants are available in the neuronal nicotinic literature) and might show whether the antagonist site (which overlaps with the ACh site) is changed by β3 (see “Discussion”).

In both receptor combinations trimetaphan (0.2–1 μM) produced a parallel rightward shift in the ACh partial concentration-response curves; its competitive mode of action was confirmed by the slope of the Schild plot (close to 1; see Table III). The incorporation of the β3 subunits had only a slight effect on the Kd for trimetaphan, decreasing it from 75.5 ± 1.8 to 66.0 ± 1.7 nM (Table III).

Although not very potent on α3β4-type receptors, the alkaloid dihydro-β-erythroidine is likely to be competitive (17). In agreement with this, we observed a small, parallel shift in the ACh concentration-response curve in the presence of 30 μM dihydro-β-erythroidine, with similar dose ratios for α3β4 and α3β4β3 (3.3 versus 3.9, n = 2), again suggesting that there is no change in the antagonist binding site.

On ganglion-type nAChRs, mecamylamine is a channel blocker at micromolar concentrations (18) but may act competitively at nanomolar concentrations (16). In our experiments, the mecamylamine concentration needed to produce a significant antagonist action was high (1 μM) and had substantial channel blocking effects (seen as a reduction in the slope of the ACh concentration-response curve); again, the effect was very similar for α3β4 and α3β4β3 receptors (data not shown).

Calcium Permeability—This was similar for the two combinations. The reversal potential shift after a 10-fold increase in extracellular calcium was 7.7 ± 1.5 and 5.2 ± 0.8 mV for α3β4 and α3β4β3, respectively (Table IV; see the value of 6.1 reported for α3β4 receptors (19).

**DISCUSSION**

Our results show that the β3 subunit consistently incorporates into neuronal nicotinic α3β4 receptors and profoundly changes their single channel properties, markedly shortening open periods and bursts and increasing single channel conductance. On the other hand, the presence of β3 had only small or negligible effects on the receptor sensitivity to a series of nicotinic agonists or to the competitive antagonists trimetaphan and dihydro-β-erythroidine and on the channel permeability to calcium. None of these changes is likely to be useful in the identification of native β3-containing receptors; the decrease in lobeline potency is small, and the increase in single channel conductance is not helpful because neuronal nAChRs display a large range of conductances, usually with several overlapping levels for each combination (13).

The main difference observed in the single-channel records of β3-containing receptors was a decrease in the duration of apparent open times and burst length. In an ideal record, i.e. with perfect resolution, the duration of open times reflects only the value of the closing rate constant, α, and therefore, the gating properties of the receptor. The open times measured from a real

---

**Table I**

| Properties of β3-containing Nicotinic Receptors | 44037 |

Data from a total of 10 α3β4 patches and 5 α3β4β3 patches are shown. Note that the areas of the different components were averaged for the total number of patches (i.e. including values of 0 for patches that did not require that particular component in the fitting of the distribution).
recording are, however, distorted by our finite temporal resolution, as undetected short gaps lengthen apparent openings. In our case, because it is likely that we missed only shutttings, we could apply an approximate correction for missed events to the apparent open times. This did not change much the effect of \( \beta_3 \) incorporation.

A potentially important consequence of the decrease in burst length we observed is that synaptic currents mediated by such channels would decay faster than those mediated by \( \alpha_3 \beta_4 \) channels. A caution is that long bursts may be a feature only of recombinant \( \alpha_3 \beta_4 \) receptors, particularly in the oocyte expression system (13, 20). On the other hand, channel openings similar to recombinant long bursts have been recorded from the medial habenula, which expresses \( \alpha_3 \) and \( \beta_4 \) subunits (21), so

---

**FIG. 3.** Co-expression of the \( \beta_3 \) subunit does not affect the potency of most agonists on \( \alpha_3 \beta_4 \) human neuronal nicotinic receptors expressed in *Xenopus* oocytes. The traces in A are examples of inward currents recorded from oocytes expressing either \( \alpha_3 \beta_4 \) (left) or \( \alpha_3 \beta_4 \beta_3 \) (right) in response to low agonist concentrations. The log-log plots in B are two typical experiments; each set of dose-response curves was obtained from a single oocyte. The curves refer to epibatidine (filled inverted triangles), DMPP (filled diamonds), cytisine (filled triangles), nicotine (filled circles), acetylcholine (filled squares) and carbachol (hollow circles). Note that the potency ranks were the same for both combinations. CCh, carbachol; Nic, nicotine; Cyt, cytisine; Epi, epibatidine.

**TABLE II**

Potency ratios of a series of nicotinic agonists on human \( \alpha_3 \beta_4 \) and \( \alpha_3 \beta_4 \beta_3 \) receptors

| Agonist | Potency ratio [95% confidence interval] | Hill slope | n | Potency ratio [95% confidence interval] | Hill slope | n |
|---------|----------------------------------------|------------|---|----------------------------------------|------------|---|
| \(+\)-Epibatidine | 5828 \pm 143.5 [5269–6518] | 1.69 \pm 0.19 | 3 | 5831 \pm 262.3 [5226–6593] | 1.72 \pm 0.08 | 6 |
| Lobeline | 23.0 \pm 3.70 [16.5–38] | 1.07 \pm 0.12 | 7 | 7.14 \pm 1.11 [5.1–11.9] | 1.18 \pm 0.08 | 6 |
| Cytisine | 3.23 \pm 0.45 [2.37–5.06] | 1.52 \pm 0.10 | 6 | 3.10 \pm 0.194 [2.69–3.66] | 1.51 \pm 0.14 | 7 |
| DMPP | 2.28 \pm 0.41 [1.58–4.07] | 1.64 \pm 0.16 | 7 | 2.21 \pm 0.057 [2.09–2.34] | 1.46 \pm 0.09 | 9 |
| Nicotine | 1.77 \pm 0.11 [1.55–2.06] | 1.49 \pm 0.10 | 9 | 1.60 \pm 0.098 [1.41–1.85] | 1.52 \pm 0.08 | 12 |
| ACh | 1.61 \pm 0.07 [1] | 1.95 \pm 0.024 [0.96–0.217] | 23 | 1.30 \pm 0.005 [0.117–0.145] | 1.50 \pm 0.04 | 27 |

\( \alpha_3 \beta_4 \) | \( \alpha_3 \beta_4 \beta_3 \)

\( \alpha_3 \beta_4 \) vs. \( \alpha_3 \beta_4 \beta_3 \) statistically significant \( p < 0.01 \), two-tailed Student \( t \) test.

---

\( \alpha_3 \beta_4 \) and \( \alpha_3 \beta_4 \beta_3 \) are two-tailed Student \( t \) test.
Fig. 4. The potency of lobeline relative to ACh is lower in receptors containing the β3 subunit. Traces (A) show the current responses to bath application of low concentrations of ACh or lobeline to α3β4 (left) or α3β4β3 (right) receptors. Concentration-response curves from such traces are plotted in B. The curves refer to lobeline, ACh, and carbachol (left to right). Note that in the experiment on triplet α3β4β3 receptors (right) the lobeline curve (filled circles) is closer to that of ACh (filled squares) than it is in oocytes expressing α3β4 (left). Lob, lobeline.

TABLE III
Schild analysis of the antagonist effect of trimetaphan on α3β4 and α3β4β3 receptors

| Subunit combination | Kᵦ (mM) | Schild slope |
|---------------------|---------|-------------|
| α3β4 (n = 10)       | 75.5 ± 1.8 | 0.80 ± 0.10 |
| α3β4β3 (n = 15)     | [71.9–79.4] | [0.60–1.00] |

TABLE IV
Effect of changes in external calcium on the reversal potential of the nicotinic current through α3β4 and α3β4β3 receptors

| Subunit combination | Reversal potential 1.8 mM Ca²⁺ | Shift in reversal potential | n |
|---------------------|---------------------|---------------------------|---|
| α3β4                | −18.7 ± 1.0         | −11.0 ± 1.9              | 5 |
| α3β4β3              | −15.1 ± 2.0         | −9.9 ± 1.4              | 5 |

it is possible that incorporation of the β3 subunit does exert this effect in native channels.

Another point of interest concerns the role of the β3 subunit in the receptor structure. Our previous work showed that α3β4β3 receptors contain two copies each of α3 and β4 and only one copy of β3 (2). Our single channel data are in good accord with this stoichiometry, as the increase in conductance with β3 incorporation strongly indicates that the β3 subunit takes the place of one of the β4 copies in the α3β4 pentamer. In nicotinic receptors conductance is mostly determined by the net charge at each of three sets of residues, at positions −4′, −1′, and 20′ of the TM2 (second transmembrane) domain, the cytoplasmic, intermediate, and outer rings of charges (22). At the cytoplasmic and intermediate rings, all the subunits we expressed in this study are similar and contribute a negative charge (aspartate or glutamate). However, at the outer ring, α3 and β3 have a negatively charged glutamate, whereas β4 has a positively charged lysine. Hence, β3 can only affect the rings of charges if it takes the place of a β4 subunit; in this case, the charge on the outer ring would become more negative by two units, explaining the substantial conductance increase we observed. For instance, it has been reported that changing lysine to glutamate in the 20′ position of one of the three copies of β2 is sufficient to increase the chord conductance of α4β2 nAChRs by ~35% in divalent-free solution and using potassium as the permeant ion (23).

Incorporation of the α5 subunit is also known to increase single channel conductance of neuronal nAChR (13, 24, 25), probably by a similar effect on the outer ring (note, however, that α5, but not β3, incorporation should also reduce by one unit the negative charge on the cytoplasmic ring). If β3 replaced an α rather than a β subunit, the only change in the residues important for conductance would be a conservative serine for threonine swap in position 2′, which in muscle nicotinic receptors produces a small increase in conductance, detectable only if potassium is the main permeant ion (22, 26). However, knowing that β3 replaces a β4 subunit still leaves open the question if β3 could participate directly, as the β, or complementary (−) subunit, to one of the two agonist binding sites at the subunit interface.

Our antagonist data discount major changes in the binding sites given that the antagonist binding was not affected by β3 (judging from the results of our Schild analysis for trimetaphan and dihydro-β-erythroidine). Of course minor changes in the agonist binding residues may not be resolvable by competitive antagonists because their binding site overlaps but does not coincide with that of the agonist (note also that the range of
competitive antagonists available is relatively small). This may well be the case for a β4 to β3 swap, as the sequences of β4 and β3 in loops D, E, and F are fairly similar; note, however, that the important residues in these domains are not as well characterized as those for loops that form the α-side of the interface (27). Additionally, β3 can in principle only change one of the binding sites, not both. The Schild method measures the dissociation constant of the highest affinity site, as antagonist occupancy of one site only is sufficient to block receptor activation (muscle nAChR with one agonist and one antagonist molecule bound have very low \( P_{\text{open}} \); see Sine and Taylor (28)). Thus, any β3-induced decrease in the site affinity for the antagonist would not be detectable.

Our data from the agonist experiments also argue against major changes in binding sites; despite testing a wide range of nicotinic agonists, the only change we observed with β3 incorporation was a decrease in the relative potency of lobeline. In receptors containing β3, there is no change in the \( EC_{50} \) of ACh or P.ACh.E (27). Additionally, the Schild method measures the dissociation constant of the highest affinity site, as antagonist occupancy of one site only is sufficient to block receptor activation (muscle nAChR with one agonist and one antagonist molecule bound have very low \( P_{\text{open}} \); see Sine and Taylor (28)). Thus, any β3-induced decrease in the site affinity for the antagonist would not be detectable.

Our data from the agonist experiments also argue against major changes in binding sites; despite testing a wide range of nicotinic agonists, the only change we observed with β3 incorporation was a decrease in the relative potency of lobeline. In principle, differences in agonist potency can be due to changes in either the binding or gating steps, which cannot be distinguished at macroscopic level (29). The analysis of open periods provides strong evidence of a gating impairment because of the shorter open times observed in β3-containing receptors. A gating change seems, therefore, to be the most likely explanation of the effects of β3 incorporation. Further work is needed to clarify why, as we previously reported, in receptors containing β3 there is no change in the \( EC_{50} \) of ACh or nicotine and in the maximum response (relative to ACh) of the partial agonist nicotine (1). Our data show that the receptor binding sites are not appreciably changed by β3 incorporation. Thus, either β3 plays the role of a “structural” subunit or its contribution to the binding site cannot be detected because the sequences of the relevant domains are too similar to those of β4. This issue may only be decided conclusively by a different strategy, such as expressing β3 after the introduction of mutations aimed at increasing binding site differences for antagonists. Designing such mutants could for instance exploit the evidence on the determinants of binding for dihydro-β-erythroidine and α-conotoxin MII provided by Luetje and co-workers (30–32).

REFERENCES
1. Groot-Kormelink, P. J., Loyten, W. H. M., Colquhoun, D., and Sivilotti, L. G. (1998) J. Biol. Chem. 273, 15317–15320
2. Boorman, J. P., Groot-Kormelink, P. J., and Sivilotti, L. G. (2000) J. Physiol. (Lond.) 529, 567–578
3. Forsayeth, J. R., and Kobrin, E. (1997) J. Neurosci. 17, 1531–1538
4. Groot-Kormelink, P. J., and Loyten, W. H. M. L. (1997) FEBS Lett. 400, 309–314
5. Akopian, A. N., Sivilotti, L., and Wood, J. N. (1996) Nature 379, 257–262
6. Quick, M. W., and Lester, H. A. (1994) Methods Neurow. 19, 261–279
7. Methfessel, C., Witzemann, V., Takahashi, T., Mishina, M., Numa, S., and Sakmann, B. (1986) Pfluegers Arch. Eur. J. Physiol. 407, 577–588
8. Colquhoun, D., and Sakmann, B. (1985) J. Physiol. (Lond.) 360, 501–517
9. Sands, S. B., Costa, A. C. S., and Patrick, J. W. (1993) Biophys. J. 65, 2614–2621
10. Coventon, P. J. O., Kojima, H., Sivilotti, L. G., Gibb, A. J., and Colquhoun, D. (1994) J. Physiol. (Lond.) 481, 27–34
11. Colquhoun, D. (1971) Lectures on Biostatistics, pp. 283–297, Clarendon Press, Oxford
12. Mathie, A., Call-Candy, S. G., and Colquhoun, D. (1991) J. Physiol. (Lond.) 439, 717–750
13. Sivilotti, L. G., McNeil, D. K., Lewis, T. M., Nussar, M. A., Schoepefer, R., and Colquhoun, D. (1997) J. Physiol. (Lond.) 500, 123–138
14. Chavez-Noriega, L. E., Crona, J. H., Washburn, M. S., Urrutia, A., Elliott, K. J., and Johnson, E. C. (1997) J. Pharmacol. Exp. Ther. 280, 346–356
15. Meyer, E. L., Xia, Y., and Kellar, K. J. (2001) Mol. Pharmacol. 60, 568–576
16. Ascher, P., Large, W. A., and Rang, H. P. (1979) J. Physiol. (Lond.) 295, 139–170
17. Xiao, Y., Meyer, E. L., Thompson, J. M., Surin, A., Wroblewski, J., and Kellar, K. J. (1998) Mol. Pharmacol. 54, 322–333
18. Ginatullin, R. A., Sokolova, E., Di Angelantonio, S., Skorinkin, A., Talantova, M., and Nistri, A. (2000) Mol. Pharmacol. 58, 778–787
19. Gerzanich, V., Wang, F., Kuryatov, A., and Lindstrom, J. (1998) J. Pharmacol. Exp. Ther. 286, 511–320
20. Lewis, T. M., Harkness, P. C., Sivilotti, L. G., Colquhoun, D., and Millar, N. (1997) J. Physiol. (Lond.) 505, 299–306
21. Connolly, J. G., Gibb, A. J., and Colquhoun, D. (1995) J. Physiol. (Lond.) 484, 87–105
22. Imoto, K., Busch, C., Sakmann, B., Mishina, M., Konno, T., Nakai, J., Bujo, H., Mori, Y., Fukuda, K., and Numa, S. (1988) Nature 335, 645–648
23. Cooper, E., Coutelier, S., and Ballivet, M. (1991) Nature 350, 235–238
24. Ramirez-Latorre, J., Yu, C. R., Qu, X., Perin, F., Karlin, A., and Role, L. (1996) Nature 380, 347–351
25. Nelsen, M. E., and Lindstrom, J. (1999) J. Physiol. (Lond.) 516, 657–678
26. Villarreal, A., Herlitze, S., Koenen, M., and Sakmann, B. (1991) Proc. R. Soc. Lond. B Biol. Sci. 243, 69–74
27. Bregel, K., van Dijk, W. J., Klaassen, R. V., Schuurmans, M., van der Oost, J., Smit, A. B., and Smit, A. C. T. K. (2001) Nature 411, 260–267
28. Sine, S. M., and Taylor, P. (1981) J. Biol. Chem. 256, 6692–6699
29. Colquhoun, D. (1998) Br. J. Pharmacol. 125, 923–947
30. Harvey, S. C., and Luetje, C. W. (1996) J. Neurosci. 16, 3798–3806
31. Harvey, S. C., Maddox, F. N., and Luetje, C. W. (1996) J. Neurochem. 67, 1953–1959
32. Harvey, S. C., McIntosh, J. M., Carter, G. E., Maddox, F. N., and Luetje, C. W. (1997) Mol. Pharmacol. 51, 336–342