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A Reporter Mutation Approach Shows Incorporation of the "Orphan" Subunit β3 into a Functional Nicotinic Receptor*

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We have investigated whether the neuronal nicotinic subunit β3 can participate in the assembly of functional recombinant receptors. Although β3 is expressed in several areas of the central nervous system, it does not form functional receptors when expressed heterologously together with an α or another β nicotinic subunit. We inserted into the human β3 subunit a reporter mutation (V273T), which, if incorporated into a functional receptor, would be expected to increase its agonist sensitivity and maximum response to partial agonists. Expressing the mutant $\beta3^{V273T}$ in *Xenopus* oocytes together with both the α3 and the β4 subunits resulted in the predicted changes in the properties of the resulting nicotinic receptor when compared with those of α3β4 receptors. This indicated that some of the receptors incorporated the mutant β3 subunit, as part of a "triplet" α3β4 β3 receptor. The proportion of triplet receptors was dependent on the ratios of the α3:β4:β3 cRNA injected. We conclude that, like the related α5 subunit, the β3 subunit can form functional receptors only if expressed together with both α and β subunits.

Molecular cloning has brought to light an unsuspected multitude of subunits that are the building blocks of ligand-gated ion channels. Our understanding of the functional importance of this diversity is still very incomplete. We need to know which subunits can co-assemble, which ones actually do so in native tissue, and whether the properties of the receptors assembled from different subunit combinations are different in a physiologically meaningful way (1).

A good example is that of the neuronal nicotinic acetylcholine (ACh)[1] receptors, for which as many as 11 different subunits (named α2–α9 and β2–β4) have been cloned. Heterologous expression has shown that, apart from α7, α8, and α9, which can form homomeric receptors, functional receptors require the co-expression of an "α/β" pair of subunits, *i.e.* α2–4 with β2 or β4 (2). The α6 subunit contributes to functional receptors if expressed with β2, with β4 or, as a "triplet," with α3 and β4 (3, 4). Neither α5 nor β3 can form α/β "pair" receptors: α5 can form functional triplet receptors with α3β2, α3β4, or α4β2 (detectable because of changes in agonist sensitivity, macroscopic desensitization, and channel conductance (5–7)). Although the β3 subunit was discovered more than 9 years ago (8), its role remains obscure. It could be that β3 is a transcribed pseudogene (*i.e.* a non-functional gene) or that the β3 subunit coassembles into a functional nicotinic receptor only with another, yet to be identified, subunit. Alternatively, it is conceivable that β3, like the α5 subunit, could form functional receptors only if expressed in a triplet combination. This is supported by the high similarity between the α5 and β3 subunit (80% amino acid sequence similarity, *i.e.* identical amino acids and conservative substitutions) and they have been classified in a separate group within the neuronal nicotinic receptor family (9).

However, the number of potential triplet combinations that would have to be screened is large, because β3 can be immunoprecipitated from at least five different brain regions, where it is present in neurones that express a variety of other subunits (2, 10, 11). Unless the effects of β3 on receptor properties are as striking as those of α5, the range of tests to be carried out is likely to be extensive. In addition, there may be differences in triplet receptor assembly between oocytes and cell lines (12), *e.g.* α3β4 receptors expressed in oocytes differ in their channel properties from α3β4 receptors expressed in HEK293 cells (13). Until it has been established which of the heterologous expression systems is closer to native neurones for each combination, it is desirable that these tests should be carried out both in oocytes and in cell lines.

We have investigated which β3-containing triplet combinations assemble by inserting into the β3 subunit a reporter mutation, V273T. This mutation converts the hydrophobic residue in the middle of the pore-lining second transmembrane domain (TM2) into a hydrophilic residue. In the nicotinic receptors in which this type of mutation has been tested (α7 (14) and mouse muscle (15, 16)), in the α1β2γ2 γ-aminobutyric acid receptor (17), and in the $5HT_3$ receptor (18), it resulted in a pronounced leftward shift of the agonist concentration-response curve. This shift was found to increase regularly with the number of mutated subunits incorporated (15) and is likely to result from changes in the gating equilibrium constant due to destabilization of the closed state, although there may also be a contribution by the desensitized state becoming conducting (14).

We found that expressing $\beta3^{V273T}$ together with the α3 and β4 subunits in *Xenopus* oocytes changes the pharmacological properties of the α3β4 recombinant nicotinic receptor, suggesting that the $\beta3^{V273T}$ subunit is incorporated into a functional $\alpha3\beta4\beta3^{V273T}$ receptor.

EXPERIMENTAL PROCEDURES

Construction of cRNA for Oocyte Expression—cDNAs for the human α3, β3, and β4 (GenBank™ accession numbers Y08418, Y08417, and Y08416, respectively), containing only coding sequences and an added Kozak consensus sequence (GCCACC) immediately upstream of the start codon (19), were subcloned into the pSP64GL vector, which con-

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[1] The abbreviations used are: ACh, acetylcholine; EC_{50}, the agonist concentration that produces 50% of the maximum response; I_{max}, the maximum response to the agonist; n_H, the Hill coefficient; TM2, second transmembrane domain.

tains 5′- and 3′-untranslated *Xenopus* β-globin regions (20). The V273T mutation was inserted in β3 using the QuickChange™ Site-directed Mutagenesis Kit (Stratagene; mutagenesis primer used: 5′-GTCGACAGAAAAGACTTCTTCGATAACGGAGAATGG-3′), and the full-length sequence was verified. All four cDNA/pSP64GL plasmids were linearized immediately downstream of the 3′-untranslated β-globin sequence, and cRNA was transcribed using the SP6 Mmessage Mmachine Kit (Ambion). The quality and quantity were checked by RNA gel electrophoresis and comparison with RNA concentration and size markers.

Expression in Xenopus Oocytes—Mature female *Xenopus laevis* frogs were anesthetized by immersion in a 0.2% solution (pH 5.6) of ethyl *m*-aminobenzoate (methanesulfonate, Tricaine, Sigma) and killed by decapitation and destruction of the brain and the spinal cord. The ovarian lobes were dissected into small clumps of 5–10 oocytes, treated with collagenase (Sigma IA; 245 collagen digestion units/ml) for 75 min, and defolliculated manually. Healthy stage V-VI oocytes were selected for injection of cRNA coding for the nicotinic subunits to be expressed (23 nl, 1.2 pg of cRNA in total per oocyte). cRNA ratios were 1:1 for $\alpha3\beta4$, 1:1:1 and 1:1:20 for $\alpha3\beta4 + \beta3^{V273T}$, and 1:1:20 for $\alpha3\beta4 + \beta3^{WT}$ injections. The injection was carried out with a Drummond Nanoject injector. Oocytes were incubated in Barth's solution at 18 °C for 2 days, then stored at 4–6 °C until needed for electrophysiological recording (up to 2 weeks later).

Electrophysiological Recording—Current responses were obtained by two-electrode voltage clamp recording at a holding potential of −70 mV (Axoclamp 2B, Axon Instrument), with electrodes filled with 3 M KCl. Agonists solutions (ACh chloride and (−)-nicotine hydrogen tartrate, both from Sigma) were freshly prepared in modified Ringer's solution from frozen aliquots of stock and bath-applied (approximately 5 ml/min) to elicit inward currents which were recorded on a chart for subsequent analysis. Agonist responses were obtained at 5-min intervals, and a standard ACh concentration (50 μM) was applied every third response. The responses to 50 μM ACh were used to correct for rundown of response amplitude during the experiment, by linear interpolation.

Solutions—Barth's solution for oocyte culture had a composition of (mM) NaCl (88), KCl (1), $MgCl_2$ (0.82), $CaCl_2$ (0.77), $NaHCO_3$ (2.4), Tris-HCl (15), with 50 units ml^{-1} penicillin and 50 μg ml^{-1} streptomycin, pH 7.4. The solution was sterilized by filtration (0.22 μm pore filter, Millex-GV, Millipore).

The modified Ringer solution for oocyte recording contained (mM): NaCl (150), KCl (2.8), $MgCl_2$ (2), HEPES (10), atropine sulfate (0.5 μM), pH 7.2 adjusted with NaOH.

Data Analysis—A full concentration-response curve to ACh was obtained for each oocyte included in this study. Analysis of the results was carried out by fitting each concentration-response curve separately (by equally weighted least squares, CvFit program by David Colquhoun) with the Hill equation $I = I_{max}(x^{n_H}/(EC_{50}{}^{n_H} + x^{n_H}))$ where I_{max} is the maximum response to the agonist, x the agonist concentration, n_H the Hill coefficient, and EC_{50} the agonist concentration that produces 50% of the maximum response.

RESULTS AND DISCUSSION

We have expressed in *Xenopus* oocytes the $\alpha3\beta4$ combination alone or together with wild type β3 ($\beta3^{WT}$) or mutant β3 ($\beta3^{V273T}$). Addition of cRNA for the $\beta3^{WT}$ subunit to α3 and β4 did not produce a detectable shift in the ACh concentration-response curve (see Fig. 1 and Table I), but when $\beta3^{V273T}$ was added instead of the $\beta3^{WT}$, a pronounced leftward shift in the ACh concentration-response curve was observed. This suggests that the mutated β3 subunit is incorporated into the expressed receptor.

The next question is, what proportion of the receptors expressed after injection of α3, β4, and β3 do actually contain β3? The results in Table I and Fig. 1 were all obtained by injection of cRNAs for these subunits in a ratio of 1:1:20. We can obtain an estimate of the proportion of current due to triplet receptors by fitting the ACh concentration-response curves with the sum of two Hill equations, the first of which is supposed to represent pair receptors and therefore has its EC_{50} and Hill slope (n_H) fixed at the values already determined in oocytes expressing $\alpha3\beta4$ alone (180 μM and 1.81, respectively (Table I)). Fig. 1*B* shows four concentration-response curves from oocytes injected with α3, β4, and $\beta3^{V273T}$ cRNAs in the ratio 1:1:1. The fit shown (see legend) gives an estimate of the fraction of current carried by triplet channels as 61 ± 4.3% (n = 4) with 2-unit likelihood intervals (roughly 95% confidence) of 54% to 73%. The EC_{50} and n_H values estimated for the mutant triplet receptor (2nd component in Fig. 1*B*) were 37.7 ± 3.6 μM and 1.31 ± 0.09 for the 1:1:1 injections with $\beta3^{V273T}$, very close to the values obtained by a two-component fit of the 1:1:20 injections (41.8 ± 6.5 μM, 1.22 ± 0.06). When the cRNAs were injected in the ratio 1:1:20, fitting two components produced only a slight improvement (0.5 log likelihood units) over a single component, so it was not possible to estimate the fraction attributable to the second component (for either $\beta3^{WT}$ or $\beta3^{V273T}$), but the results were consistent with a large proportion of the receptors being triplets.

A change in gating for receptors containing the mutant subunit would also be expected to change the agonist efficacy, the maximum open probability, and therefore the maximum

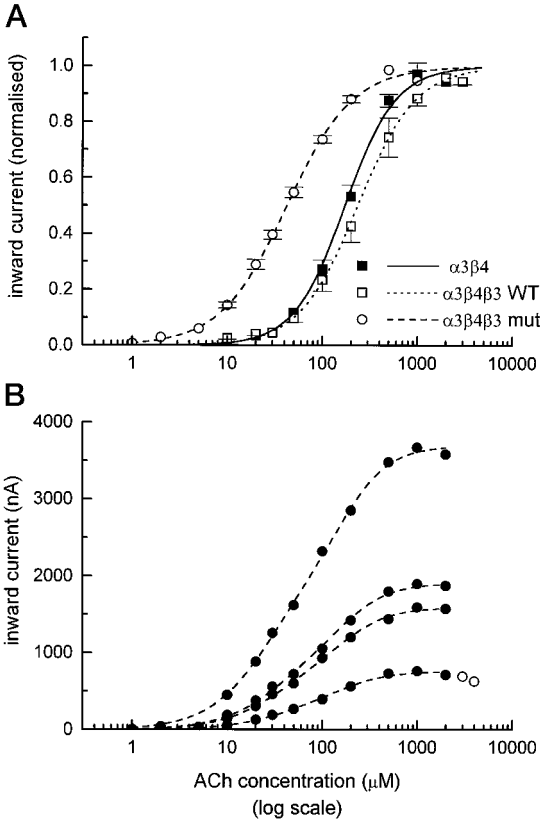

FIG. 1. **The V273T mutation in the TM2 of β3 produces a marked increase in the sensitivity to ACh of oocytes injected with $\alpha3\beta4 + \beta3^{V273T}$.** Responses are peak inward currents elicited by bath application of ACh to oocytes clamped at −70 mV in modified Ringer solution with no added calcium. The curves declined at higher concentrations, and some high concentration points (*open symbols* in *B*) have been arbitrarily omitted from the fit. Results were fitted with the Hill equation (see "Experimental Procedures"). *A*, the α3, β4, β3 cRNAs were injected in a ratio of 1:1:20 for both $\beta3^{WT}$ and $\beta3^{V273T}$. For display all responses for each subunit combination were pooled after each response had been normalized to the fitted maximum for each individual oocyte (CvFit program, n = 7, 5, and 9 for $\alpha3\beta4$, $\alpha3\beta4\beta3^{WT}$, and $\alpha3\beta4\beta3^{V273T}$, respectively). The values in Table I were obtained by averaging estimates found by fitting separate concentration-response curves, but direct fitting of the pooled curves shown here gives results that are not greatly different. *B*, two-component fits to concentration-response curves on four oocytes injected with α3, β4, $\beta3^{V273T}$ cRNAs in a ratios of 1:1:1. The four curves were fitted simultaneously with the sum of two Hill equations. There were 10 free parameters, namely the maximum response for each curve, the EC_{50} for the second component (that presumed to represent triplet receptors) for each curve, the Hill coefficient for the second component (assumed to be the same for all curves), and the fraction of the total maximum response attributable to the second component (also assumed to be the same for all curves).

TABLE I

Effect of the V273T β3 mutation on concentration-response curves to ACh and nicotine in oocytes

Parameter estimates were obtained by fitting separately the individual concentration-response curves. Values that were well defined were then used to find the means ± S.D. shown in the table.

	ACh			Nicotine			
	EC_{50} (*n*)	I_{max}	n_H	EC50 (*n*)	I_{max}	I_{max} (% I_{max} for ACh)	n_H
	μM	*nA*		*μM*	*nA*		
α3β4	180 ± 17.0 (7)	1430 ± 425 (7)	1.81 ± 0.09 (7)	70.3 ± 9.0 (5)	414 ± 146 (5)	44.7 ± 2.3 (5)	1.98 ± 0.14 (5)
α3β4 + $β3^{WT}$	258 ± 50.6 (5)	1510 ± 404 (5)	1.50 ± 0.06 (5)	95.9 ± 21.0 (5)	617 ± 175 (5)	39.6 ± 3.3 (5)	1.70 ± 0.23 (5)
α3β4 + $β3^{V273T}$	42.6 ± 2.3 (9)	214.4 ± 40.3 (5)	1.25 ± 0.04 (9)	19.4 ± 0.9 (5)	166 ± 54.4 (5)	73.0 ± 1.1 (5)	1.40 ± 0.06 (3)

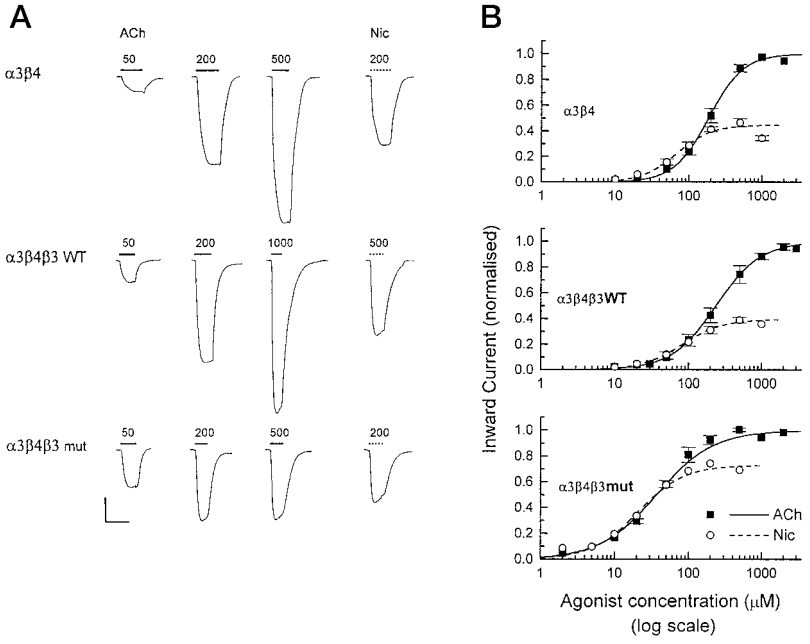

FIG. 2. **Incorporation of the β3 subunit into a functional nicotinic receptor is also demonstrated by the increase in the maximum response to nicotine in oocytes injected with α3β4 + $β3^{V273T}$.** *A*, responses to increasing concentrations of ACh and to a near-maximum concentration of nicotine, in oocytes injected with α3β4, α3β4 + $β3^{WT}$, or α3β4 + $β3^{V273T}$. *Calibration bars* are 50 nA and 40 s. *B*, concentration-response curves to ACh (*solid squares*) and nicotine (*open circles*) in oocytes injected with α3β4, α3β4 + $β3^{WT}$ or α3β4 + $β3^{V273T}$ ($n = 5$ for each combination; cRNA injection ratio 1:1:20 for both $β3^{WT}$ and $β3^{V273T}$). Each oocyte provided an ACh and a nicotine concentration-response curve. Data were fitted as in Fig. 1, after normalization to the fitted maximum of the ACh concentration-response curve in each oocyte. Note the increase in the maximum response to nicotine (relative to the maximum ACh response) for α3β4 + $β3^{V273T}$ (see Table I).

agonist response. Whether the change in maximum response is big enough to be detected will depend on whether efficacy increases or decreases and on the agonist efficacy in the wild-type receptor. If the mutation shifts the equilibrium toward the open state of the bound receptor (as in our case), efficacy should increase, but an increase in the maximum agonist response would be noticeable only if the wild-type maximum open probability was well below 1, *i.e.* if the agonist is a partial agonist (21). While little is known of the actual efficacy of nicotinic agonists on neuronal receptors, nicotine is a good example of a possible partial agonist on α3β4, as it elicits a maximum response which is only 44.7 ± 2.3% of that to ACh (see Fig. 2). The maximum response to nicotine (as a fraction of the ACh maximum) is nearly doubled by the mutation, fulfilling the prediction (see α3β4$β3^{WT}$ *versus* α3β4 $β3^{V273T}$ in Table I and in Fig. 2). Clearly, changes in agonist channel block and in desensitization (following the V273T mutation) may contribute to this effect, and further work is necessary to exclude this possibility.

We have shown that the β3 subunit can be incorporated into a functional α3β4β3 recombinant nicotinic receptor. While β3 is expressed in several areas of the nervous system which lack α3 and β4, the β3 subunit is also abundant in sensory ganglia, which are rich in α3 and β4 subunits. The nicotinic receptors of sensory ganglia (which are potential targets for nicotinic analgesia (22)) may therefore have an α3β4β3 composition. The reporter mutation approach used here will allow rapid screening of other subunit combinations containing β3 and will establish which of these can form functional receptors. In addition, the optimal cRNA ratios needed to ensure that the majority of assembled receptors are triplets can be determined. Once combination and optimal conditions have been identified by expression of the mutant, the wild-type triplet can be expressed heterologously to characterize the pharmacological and biophysical properties of the resulting receptor. Furthermore, if in neuronal nicotinic receptors, as in muscle receptors, the EC_{50} shift produced by the hydrophilic mutation in the middle of TM2 is proportional to the number of mutated subunits (15, 16), this approach can also be extended to the question of the stoichiometry of triplet receptors. Finally, as the mid-TM2 Leu (or Val) motif is present in all subunits belonging to the 4-transmembrane domain receptor superfamily, the method can readily be extended to other receptor types.

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