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Formation of functional $\alpha3\beta4\alpha5$ human neuronal nicotinic receptors in *Xenopus* oocytes: a reporter mutation approach

[1]Paul J. Groot-Kormelink, [1]James P. Boorman & *,[1]Lucia G. Sivilotti

[1]Department of Pharmacology, The School of Pharmacy, 29/39 Brunswick Square, London WC1N 1AX

1 The $\alpha5$ subunit participates to the formation of native neuronal nicotinic receptors, particularly in autonomic ganglia. Like the related $\beta3$ subunit, $\alpha5$ forms functional recombinant receptors if expressed together with a pair of typical α and β subunits, but its effect on the properties of the resulting $\alpha\beta\alpha5$ receptor depends on the α and β subunits chosen and on the expression system. We used a reporter mutation approach to test whether $\alpha5$, like $\beta3$, is incorporated as a single copy in human $\alpha3\beta4\alpha5$ receptors expressed in oocytes.

2 As previously reported, the main indication of the presence of $\alpha5$ in $\alpha3\beta4\alpha5^{wt}$ was an increase in apparent receptor desensitization (compared with $\alpha3\beta4$ receptors). If the $\alpha3\beta4\alpha5$ receptor bore a 9′T mutation in the second transmembrane domain of either $\alpha3$ or $\beta4$, $\alpha5$ incorporation produced a decrease in ACh sensitivity (by 4 fold for $\alpha3^{LT}\beta4\alpha5$ *vs* $\alpha3^{LT}\beta4$ and by 40 fold for $\alpha3\beta4^{LT}\alpha5$ *vs* $\alpha3\beta4^{LT}$). The much greater effect observed in $\alpha3\beta4^{LT}\alpha5$ receptors accords with the hypothesis that $\alpha5$ takes the place of a β subunit in the receptor.

3 Introducing a 9′T mutation in $\alpha5$ had no effect on the agonist sensitivity of $\alpha3\beta4\alpha5$ receptors, but reduced apparent desensitisation, as judged by the sag in the current response to high agonist concentrations.

4 Introducing the 9′T mutation in $\alpha3$ or $\beta4$ in the triplet receptor reduced the EC_{50} for ACh by a similar extent (7 and 9 fold, respectively), suggesting that $\alpha3\beta4\alpha5$ receptors contain two copies each of α and β and therefore only one copy of $\alpha5$.

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Abbreviations: ACh, acetylcholine; nAChR nicotinic acetylcholine receptor; TM2, second transmembrane domain

Introduction

When the $\alpha5$ neuronal nicotinic subunit was cloned (Boulter *et al*., 1990; Couturier *et al*., 1990), it was classified as an α subunit, because of the presence of two adjacent cysteines in the first extracellular domain. Nevertheless, $\alpha5$ differs from typical α subunits in the domains thought to be important for agonist binding, lacking, for instance, Tyr93 and Tyr190. In nicotinic ACh receptors (nAChR) typical α subunits contribute either the whole of the agonist binding domain (Unwin, 2001) or the subsite for the ACh ester moiety (Karlin & Akabas, 1995; Brejc *et al*., 2001). A typical neuronal α subunit ($\alpha2$, $\alpha3$ or $\alpha4$) will form functional nAChRs when expressed heterologously together with a typical β subunit ($\beta2$ or $\beta4$; Lindstrom, 2000). These pentameric complexes contain two copies of the α and three copies of the β subunit (Cooper *et al*., 1991; Boorman *et al*., 2000). A few α subunits ($\alpha7$, $\alpha8$ and $\alpha9$) can form functional homomeric nAChRs when expressed alone in *Xenopus* oocytes.

Despite its classification, $\alpha5$ behaves differently from typical α subunits, and cannot form a functional receptor when expressed alone or together with a β subunit (Boulter *et al*., 1990). The inability of $\alpha5$ to produce functional recombinant receptors was puzzling because $\alpha5$ forms one of the main synaptic nAChRs in chick ciliary ganglia (with $\alpha3$ and $\beta4$, Conroy & Berg, 1995; Vernallis *et al*., 1993) and contributes to more than one nAChR subtype in chick sympathetic neurones (Yu & Role, 1998). In chick optic lobe $\alpha5$ is also known to assemble with $\alpha2$ and $\beta2$ (Balestra *et al*., 2000) and with $\alpha3$ and $\beta2$ in human SH-SY5Y neuroblastoma cells (Wang *et al*., 1996).

This apparent contradiction was resolved when Ramirez-Latorre *et al*. (1996) and Wang *et al*. (1996) showed that $\alpha5$ contributes to functional recombinant nAChRs only if it is expressed together with both an α and a β subunit, such as $\alpha4\beta2$, $\alpha3\beta2$ or $\alpha3\beta4$, in a triplet form. Incorporation of the $\alpha5$ subunit was detected because a new receptor population appeared, considerably less sensitive to the agonist action of ACh. These $\alpha5$-containing nAChRs also have a higher single channel conductance than the 'parent' pair receptors (Ramirez-Latorre *et al*., 1996; Sivilotti *et al*., 1997; Nelson & Lindstrom, 1999). Lindstrom and co-workers however showed that the effect of the presence of $\alpha5$ depends on the nature of the 'parent' pair of subunits with which $\alpha5$ is co-expressed. For instance, in oocytes $\alpha5$ markedly increased the ACh sensitivity of human $\alpha3\beta2\alpha5$ receptors, but left unchanged that of $\alpha3\beta4\alpha5$ nAChRs (Wang *et al*., 1996; Gerzanich *et al*., 1998). In $\alpha3\beta4\alpha5$ nAChRs, $\alpha5$ incorporation is manifest because of changes in the desensitization and calcium permeability of the receptor. On the other hand, if chick subunits are expressed, $\alpha5$ decreases $\alpha3\beta4$ agonist sensitivity in mammalian cells, but has no effect if these

*Author for correspondence; E-mail: Lucia.Sivilotti@ulsop.ac.uk

subunits are expressed in oocytes (Fucile *et al*., 1997). Indeed, these data have been taken to indicate lack of co-assembly of this particular 'triplet' receptor in oocytes.

In view of such inconsistent effects of α5, it is unclear whether α5 participates, directly or indirectly, to the agonist binding site or is a structural subunit (like β1 in muscle nAChRs). Also, we know little of the activation properties of neuronal nAChRs and therefore cannot interpret unambiguously the effects of α5 on the ACh EC_{50} in terms of binding or gating effects (Colquhoun, 1998).

We recently addressed the detection of subunit incorporation for the β3 neuronal nicotinic subunit: this participates to α3β4β3 receptors as a single copy (Boorman *et al*., 2000). These findings are relevant to α5 nAChRs because β3 is the nicotinic subunit with the highest sequence similarity compared to α5 (and is classed with α5 in tribe III-3 of the evolutionary nicotinic tree, Corringer *et al*., 2000) and it forms functional nAChRs only when expressed with both an α and a β subunit, i.e. in a triplet form. Here we extend the reporter mutation strategy used for β3 to nAChRs containing α5. Our data not only confirm the functional incorporation of α5 into α3β4α5 receptors expressed in oocytes, but also show that the effect of the 9′ reporter mutation on agonist potency is much greater when α3 or β4 bear the mutation, rather than α5. The most parsimonious explanation of our results is that, similarly to recombinant α3β4β3 receptors, oocyte-expressed α3β4α5 nAChRs contain two copies each of α3 and β4, but only one copy of α5.

Methods

Construction of cRNA for oocyte expression

cDNAs for the human α3, α5, and β4 (GenBank accession numbers Y08418, Y08419 and Y08416, respectively), containing only coding sequences and an added Kozak consensus sequence (GCCACC) immediately upstream of the start codon (Groot-Kormelink & Luyten, 1997), were subcloned into the pSP64GL vector, which contains 5′ and 3′ untranslated *Xenopus* β-globin regions (Akopian *et al*., 1996). The mutants in 9′ ($\alpha3^{L279T}$, $\alpha5^{V290L}$, $\alpha5^{V290T}$ and $\beta4^{L272T}$, where V, L and T stand for valine, leucine and threonine, respectively) were created using the QuickChange™ Site-Directed Mutagenesis Kit (Stratagene) and their full-length sequence was verified. These mutants shall be referred to hereafter as $\alpha3^{LT}$, $\alpha5^{VL}$, $\alpha5^{VT}$ and $\beta4^{LT}$. All 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 of cRNAs were checked by gel-electrophoresis and comparison with RNA concentration and size markers.

Xenopus *oocyte preparation and electrophysiological recording*

Female *Xenopus laevis* frogs were anaesthetized by immersion in neutralized ethyl m-aminobenzoate solution (tricaine, methanesulphonate salt; 0.2% solution w v^{-1}; Sigma Chemical Co.), and killed by decapitation and destruction of the brain and spinal cord (in accordance to Home Office guidelines) before removal of ovarian lobes. Clumps of stage V-VI oocytes were dissected in a sterile modified Barth's solution of composition (in 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 adjusted with NaOH. The dissected oocytes were treated with collagenase (type IA, Sigma Chemical Co.; 65 minutes at 18°C, 245 collagen digestion units ml^{-1} in Barth's solution, 10–12 oocytes per ml), rinsed, stored at 4°C overnight, and manually defolliculated the following day before cRNA injection (46 nl per oocyte). The oocytes were incubated for approximately 60 h at 18°C in Barth's solution containing 5% heat-inactivated horse serum (Gibco BRL; Quick & Lester, 1994) 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 : β4 : α5) in order to express α3β4α5 receptors. A 1 : 1 : 20 ratio ensures that at least 90% of the current is carried by triplet receptors, rather than by pair α3β4 receptors. This estimate was obtained for the $\alpha3\beta4^{LT}\alpha5$ combination, see below and the Results section. The total amount of cRNA to be injected (in 46 nl of RNAse-free water) for each combination was determined empirically with the aim of achieving a maximum ACh-evoked current of 1–2 μA and was 0.25–10 ng per oocyte (see Table 1). In principle, it would be desirable to match expression levels for the different combinations, rather than maximum currents: this is difficult to achieve, as it requires independent measurement of the number of functional surface receptors.

Oocytes, held in a 0.2 ml bath, were perfused at 4.5 ml min^{-1} with modified Ringer solution (mM: NaCl 150, KCl 2.8, HEPES 10, $MgCl_2$ 2, atropine sulphate 0.5 μM, Sigma Chemical Co.; pH 7.2 adjusted with NaOH) and voltage clamped at −70 mV, using the two-electrode clamp mode of an Axoclamp-2B amplifier (Axon Instruments). Electrodes were pulled from Clark borosilicate glass GC150TF (Warner Instrument Corporation) and filled with 3 M KCl. The electrode resistance was 0.5–1 MΩ on the current-passing side. Experiments were terminated if the total holding current exceeded 2 μA, in order to reduce the effect of series resistance errors. We chose a nominally calcium-free solution in order to minimize the contribution of calcium-gated chloride conductance; this is endogenous to the *Xenopus* oocyte and may be activated by calcium entry through the neuronal nicotinic channels (see for an example Sands & Barish, 1991).

The agonist solution (acetylcholine chloride, Sigma Chemical Co.; freshly prepared from frozen stock aliquots) was applied *via* the bath perfusion, for a period sufficient to obtain a stable plateau response (at low concentrations) or the beginning of a sag after a peak (at the higher concentrations); the resulting inward current was recorded on a flat bed chart recorder (Kipp & Zonen) for later analysis. An interval of 5 min was allowed between ACh applications, as this was found to be sufficient to ensure reproducible responses (except for $\alpha3\beta4\alpha5^{VL}$, for which a 10-min interval had to be chosen to allow recovery from desensitization). In order to compensate for possible decreases in agonist sensitivity throughout the experiment, a standard concentration of ACh (approximately EC_{20} for the

Table 1 Properties of nAChRs obtained by expressing wild type and mutant α3, β4 and α5 human subunits

	EC_{50} (μM)	I_{max} (nA)	n_H	n	*Total cRNA injected* (ng)
$\alpha3\beta4$	180±17.0	1430±425	1.81±0.09	7*	0.5–1
$\alpha3\beta4\alpha5^{wt}$	207±25.6	1098±245	1.39±0.08	5	5–10
$\alpha3\beta4\alpha5^{VL}$	206±15.5	966±367	1.47±0.05	5	5–10
$\alpha3\beta4\alpha5^{VT}$	183±11.0	646±97.8	1.19±0.08	7	1–5
$\alpha3^{LT}\beta4$	5.8±1.0	1043±411	1.15±0.08	6*	0.5
$\alpha3^{LT}\beta4\alpha5^{wt}$	22.7±2.02	1122±197	1.40±0.07	4	1
$\alpha3^{LT}\beta4\alpha5^{VL}$	17.6±1.90	491±104	1.08±0.04	4	1
$\alpha3^{LT}\beta4\alpha5^{VT}$	18.2±1.00	1215±280	1.45±0.05	4	0.5
$\alpha3\beta4^{LT}$	0.75±0.05	562±264	0.92±0.08	4*	0.25–0.5
$\alpha3\beta4^{LT}\alpha5^{wt}$	30.7±1.63	2546±518	1.36±0.10	6	0.5
$\alpha3\beta4^{LT}\alpha5^{VL}$	29.2±1.57	792±209	1.31±0.03	5	0.5
$\alpha3\beta4^{LT}\alpha5^{VT}$	31.1±1.40	1476±450	1.63±0.12	4	0.25

EC_{50}, I_{max} and n_H are the means (±s.d. of the mean) of parameters estimated by fitting separately each concentration response curve with a Hill equation (see Methods). *Data for the $\alpha3\beta4$ pair combinations are reproduced for reference from Boorman *et al*., 2000

particular combination used) was applied every third response. The experiment was started 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 shown in the study are compensated for the response rundown. However, applying this compensation did not affect the conclusions of our work, as the results of analysing the original data (without compensation) were similar (data not shown).

Experimental controls

Injection of one subunit alone (α3, $\alpha3^{LT}$, α5, $\alpha5^{VL}$, $\alpha5^{VT}$, β4 or $\beta4^{LT}$, 10 ng each) or of 'pair' combinations of α5 with either α3 (α3α5, $\alpha3\alpha5^{VL}$, $\alpha3\alpha5^{VT}$, 10 ng total) or β4 (β4α5, $\beta4\alpha5^{VL}$, $\beta4\alpha5^{VT}$, 10 ng total) did not result in the expression of functional nAChRs (i.e. current responses to 1 mM ACh were less than 5 nA).

'Pair' combinations containing the $\alpha3^{LT}$ subunit together with α5 responded to 1 mM ACh with inward currents which were relatively small (maximum responses were 149±47 and 142±50 nA, $n=5$ for $\alpha3^{LT}\alpha5$ and $\alpha3^{LT}\alpha5^{VL}$, respectively). Only the $\alpha3^{LT}\alpha5^{VT}$ combination produced a larger inward current (362±145 nA, $n=5$, total cRNA injected 10 ng; approximate EC_{50} 800 μM, $n=1$). Note that the corresponding triplet combination, $\alpha3^{LT}\beta4\alpha5^{VT}$ produced a much larger average maximum current (1215±280 nA) in response to a lower ACh concentration (200 μM, EC_{50} 18.2 μM, 0.5 ng total cRNA injected, see Table 1).

$\beta4^{LT}$ combinations with α5, $\alpha5^{VL}$ or $\alpha5^{VT}$ responded to 1 mM ACh with small outward currents (less than or equal to 55 nA, $n=5$; total cRNA injection 10 ng; 1 : 1 ratio for all pair wise combinations).

Curve fitting

All dose-response curves were fitted with the Hill equation:

$$I = I_{max}\frac{[A]^{n_H}}{[A]^{n_H} + EC_{50}{}^{n_H}}, \quad (1)$$

where I is the response, measured at its peak, [A] is the agonist concentration, I_{max} is the maximum response, EC_{50} is the agonist concentration for 50% maximum response and n_H is the Hill coefficient. We used least squares fitting by the program CVFIT, courtesy of D. Colquhoun & I. Vais, available from http://www.ucl.ac.uk/Pharmacology/dc.html.

Fitting was done in stages, as follows. Each dose-response curve was fitted separately, individual responses being equally weighted, in order to obtain estimates for I_{max}, EC_{50} and n_H. The means and standard deviation of the means for each combination are shown in Table 1.

Each response in a particular oocyte was then normalized to the fitted I_{max} for that experiment; all the normalised responses for a given combination were then pooled, giving the datapoints shown by the symbols in Figures 1–4. The pooled normalized datapoints were then fitted again with the Hill equation (with weight given by the reciprocal of their variance) in order to obtain the fitted curves displayed in the figures.

In order to assess the sensitivity and reliability with which our fitting and data analysis routines would detect the presence of two different receptors and hence two distinct components in our concentration-response data, we carried out some simulations. This was done for the $\alpha3\beta4^{LT}\alpha5$ receptor type, with the aim of testing whether we would be able to detect the presence of a residual population of pair $\alpha3\beta4^{LT}$ receptors by analysis of the concentration-response data. This subunit combination should be optimal for this purpose, as it displayed the largest shift in EC_{50} following addition of α5 (44 fold, see Table 1). The program we used (HILLSIM, courtesy of David Colquhoun, UCL) produces simulated dose-response data, fits them with a one-component or a two-component Hill equation and compares the goodness of fit obtained by the two different fits. The simulated data were produced using the EC_{50} and Hill slopes we measured for $\alpha3\beta4^{LT}$ and $\alpha3\beta4^{LT}\alpha5$ (Table 1) and adding Gaussian errors (estimated from the maximum error observed in our experiments) to the simulated datapoints. Clearly, if the triplet receptors were contaminated with pair receptors, the EC_{50} we measured will be an underestimate of the EC_{50} of the true pure triplet population. Nevertheless, the distortion introduced by this would only underestimate the

sensitivity of our methods. As our aim was to check what was the minimum proportion of residual pair current that could be detected, we obtained 1000 simulated datasets for each of several different proportions of pair:triplet current (i.e. 1:99, 5:95, 10:90 etc). The goodness of fit of the two models tested (i.e. one component vs two components) was judged by calculating the Akaike information criterion (AIC) for each of the two fits to each simulated dataset:

$$\mathrm{AIC} = -2L_{\max} + 2n_{\mathrm{par}} \quad (2)$$

where $L_{\max}$ is the maximum of the log-likelihood of a fit and n_{par} is the number of free parameters in the fit. A negative value for the difference between the AIC value for the one-component fit and the two-component fit (i.e. AIC(1) − AIC(2)) was taken to indicate that a one component fit was preferable to the two component fit for that particular dataset.

Results

Co-expression of wild-type α5 does not affect the ACh sensitivity of α3β4 nAChRs

As previously reported by Wang *et al*. (1996), expressing the α5 subunit together with α3 and β4 had no apparent effect on the α3β4 ACh dose-response curve (Table 1). Both the EC_{50} and the Hill slope of α3β4 + α5 receptors were similar to those of α3β4 nAChR. Nevertheless, the time course of ACh responses recorded from oocytes expressing all three subunits was clearly different from that of α3β4 receptors. Such 'triplet' responses (shown in Figure 1A) had a much faster 'sag' to the sustained application of high ACh concentrations (indicating faster desensitization, as noted by Wang *et al*. (1996) and Gerzanich *et al*. (1998)).

Mutation of the 9′ valine in the α5 TM2 changes the time course of ACh responses, but does not change the receptor sensitivity to ACh

In order to confirm the incorporation of α5 and to establish what proportion of current, if any, is due to 'pair' nAChRs without α5, we inserted a reporter mutation in the middle of the TM2 of α5 (as we did for the β3 subunit; Boorman *et al*., 2000). All subunits belonging to the nicotinic superfamily bear a hydrophobic amino acid in this position (termed 9′), namely a leucine or (in the case of α5 and β3) a valine.

Mutation of this 9′ residue to a hydrophilic amino acid, such as threonine or serine, is known to increase the agonist sensitivity (Revah *et al*., 1991). The magnitude of this increase is proportional to the number of copies of the mutation incorporated into the receptor for muscle nAChRs (Labarca *et al*., 1995) and for neuronal nAChRs (Boorman *et al*., 2000). Surprisingly, we found that inserting a V9′T mutation in α5 had little effect on the properties of ACh receptors produced by oocytes injected with α3, β4 and $\alpha5^{VT}$. Such receptors were indistinguishable from either wild-type 'triplet' receptors or from 'pair' α3β4 receptors in their sensitivity to ACh (Figure 1, Table 1). The only noticeable difference was in the time course of the responses to high ACh concentrations, which, in the VT mutant, lost their fast desensitization (see Figure 1A). This is particularly noticeable

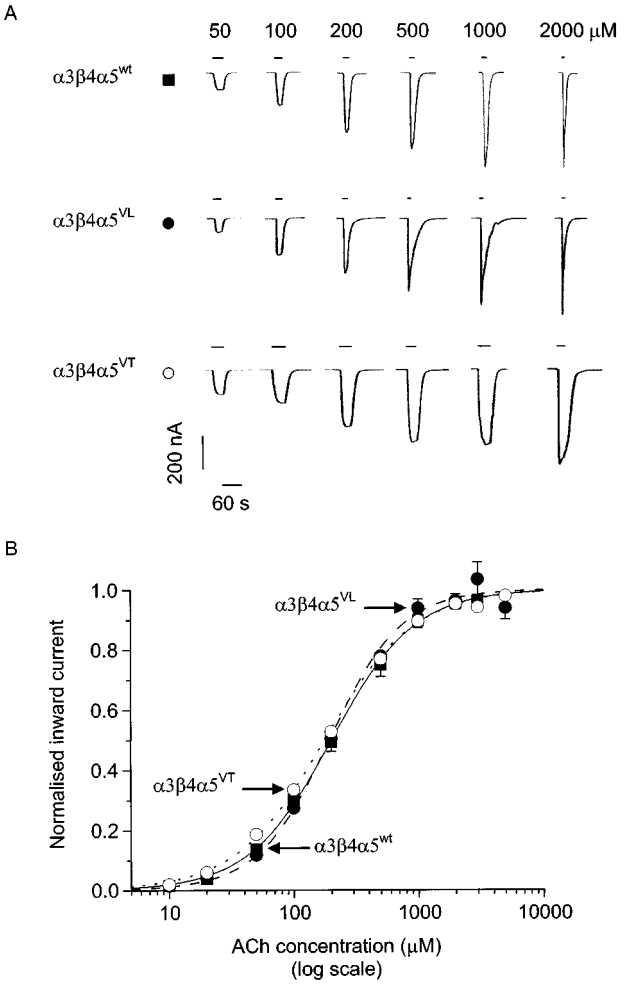

Figure 1 'Triplet' α3β4α5 nicotinic receptors containing 9′L ($\alpha3\beta4\alpha5^{VL}$) or 9′T ($\alpha3\beta4\alpha5^{VT}$) mutations in α5 are indistinguishable from the wild type form ($\alpha3\beta4\alpha5^{wt}$) in their sensitivity to ACh. (A) Examples of inward currents elicited by bath-applied ACh in oocytes expressing $\alpha3\beta4\alpha5^{wt}$, $\alpha3\beta4\alpha5^{VL}$ or $\alpha3\beta4\alpha5^{VT}$. (B) ACh concentration-response curves pooled from experiments such as the ones shown in A ($n = 5-7$). The concentration-response curves refer to oocytes injected with $\alpha3\beta4\alpha5^{wt}$ (■), $\alpha3\beta4\alpha5^{VL}$ (●) and $\alpha3\beta4\alpha5^{VT}$ (○). Curves were fitted with the Hill equation and normalized to the fitted maximum response before pooling (see Methods).

if $\alpha3\beta4\alpha5^{VT}$ receptors are compared to $\alpha3\beta4\alpha5^{VL}$ receptors. The latter bear only leucine residues in the 9′ position, have ACh sensitivity very similar to that of α3β4 or $\alpha3\beta4\alpha5^{wt}$ receptors, but show a particularly fast sag in their responses to the higher concentrations of ACh. This sag was also apparent at relatively low ACh concentrations (cfr the responses to 500 μM ACh for the wild type triplet with those of the $\alpha3\beta4\alpha5^{VL}$ receptor, Figure 1A). Thus the only noticeable effect of the presence of the α5 subunit was a change in the shape of the response to high agonist concentrations, irrespective of the nature of the residue in the 9′ position of α5. Table 1 shows that there were differences in the maximum current recorded at high agonist concentrations from the different combinations. It is impossible to interpret these differences unambiguously, as the maximum response depends on the maximum open probability, the total number of receptors and the single channel conductance of the nAChR, each of which may differ

for different combinations and for mutant vs wild-type receptors. This is particularly true of the maximum open probability, which is determined for each receptor type by the values of the gating rate constants, together with the extent of desensitization and agonist channel block at the time current peaks under our experimental conditions. There may also be differences in the total number of functional receptors on the surface, a parameter for which we do not have an independent estimate.

Co-expression of $\alpha5^{WT}$ decreases the ACh sensitivity of 9′T mutant α3β4 receptors

Because of the lack of effect of α5 on the dose-response curve to ACh, it has been argued that α5 does not form functional α3β4α5 triplet receptors in oocytes (Fucile *et al*., 1997). It would therefore be useful to have evidence of α5 incorporation in addition to the changes observed in the desensitisation time course of agonist responses. Such evidence comes from the experiments shown in Figures 2 and 3. These consist of ACh dose-response curves obtained from oocytes in which α5 was expressed together with the α3 and β4 subunits bearing a 9′T mutation in either α3 (Figure 2) or β4 (Figure 3). In both cases addition of α5 to the subunits expressed had a very pronounced effect on the dose-response curves to ACh. These were markedly shifted to the right (with respect to the mutated α3β4), confirming that these responses are not produced by α3β4 receptors, but by functional α3β4α5 receptors. The effect of adding α5 was quite prominent and occurred regardless of whether α5 was expressed in its wild type or mutant form. This would suggest that the presence of the 9′ mutations in α5 does not affect its incorporation into the receptor complex. Addition of $\alpha5^{wt}$, $\alpha5^{VL}$ or $\alpha5^{VT}$ to mutant α3β4 receptors shifted the dose-response curve to ACh to the right by approximately the same amount. This shift was approximately 3 fold for receptors bearing the 9′T mutation in α3 (from 5.8 ± 1.0 μM without α5 to 18–23 μM with α5, Figure 2 and Table 1), but exceeded 30 fold for receptors bearing the 9′T mutation in β4 (from 0.75 ± 0.05 to 29–30 μM, respectively; see Figure 3 and Table 1).

The magnitude of the EC_{50} difference between $\alpha3\beta4^{LT}$ and $\alpha3\beta4^{LT}\alpha5$ receptors allowed us to assess the maximum proportion of current due to a residual population of pair receptors. We tried to detect the presence of such pair receptors by fitting our $\alpha3\beta4^{LT}\alpha5$ data with two components

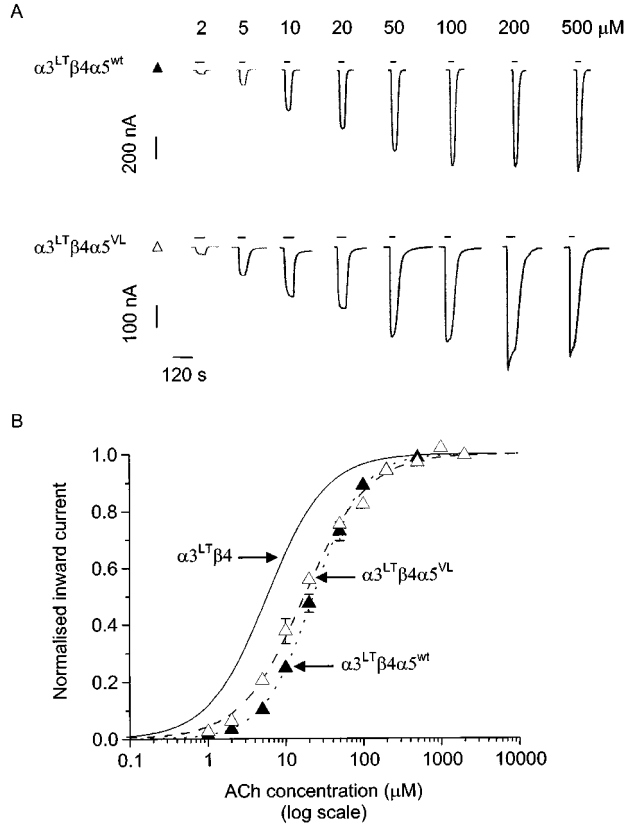

Figure 2 Addition of α5 increases the ACh EC_{50} of oocytes injected with $\alpha3^{LT}\beta4$. (A) Examples of inward currents elicited by bath-applied ACh in oocytes expressing $\alpha3^{LT}\beta4\alpha5^{wt}$ and $\alpha3^{LT}\beta4\alpha5^{VL}$. (B) ACh concentration-response curves ($n=4-6$) for oocytes injected with $\alpha3^{LT}\beta4\alpha5^{wt}$ (▲) and $\alpha3^{LT}\beta4\alpha5^{VL}$ (△) fits as in Figure 1. (B) Shows for reference (solid line without symbols) the concentration-response curve of $\alpha3^{LT}\beta4$ receptors (reproduced from Boorman *et al*., 2000). The increase in ACh EC_{50} (between 3 and 4 fold, irrespective of whether $\alpha5^{wt}$ or $\alpha5^{VL}$ is expressed) suggests the incorporation of α5 into a functional α3β4α5 'triplet' nicotinic receptor.

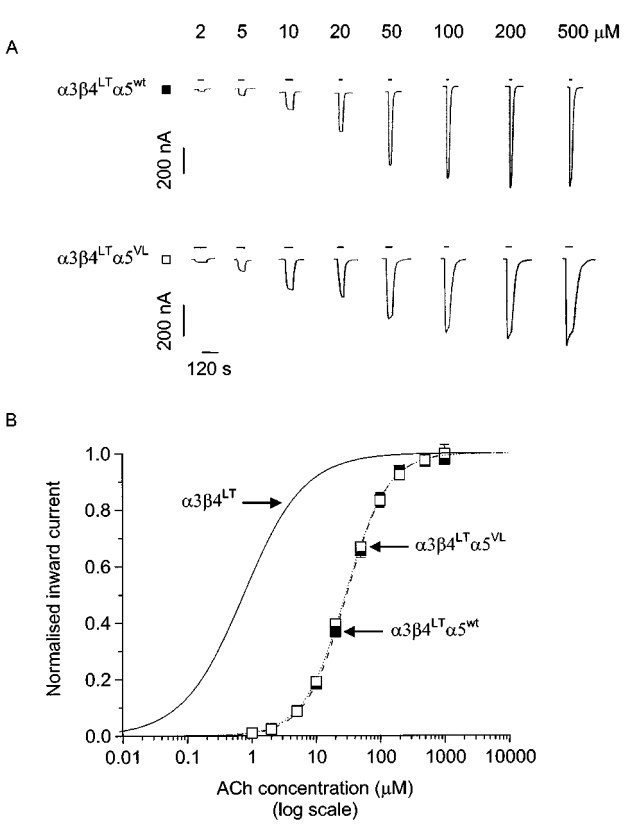

Figure 3 Addition of α5 markedly increases the ACh EC_{50} of oocytes injected with $\alpha3\beta4^{LT}$. (A) Examples of inward currents elicited by bath-applied ACh in oocytes expressing $\alpha3\beta4^{LT}\alpha5^{wt}$ and $\alpha3\beta4^{LT}\alpha5^{VL}$. (B) ACh concentration-response curves pooled from experiments such as the ones shown in A ($n=4-6$). The solid curve without symbols is the $\alpha3\beta4^{LT}$ concentration-response curve (reproduced for reference from Boorman *et al*., 2000), ■ indicate $\alpha3\beta4^{LT}\alpha5^{wt}$ and □ $\alpha3\beta4^{LT}\alpha5^{VL}$. Note that the effect of α5 on $\alpha3\beta4^{LT}$ injected oocytes is 10 fold greater than that on $\alpha3^{LT}\beta4$ (see Figure 2.), suggesting that α5 replaces a β subunit in the formation of the 'triplet'.

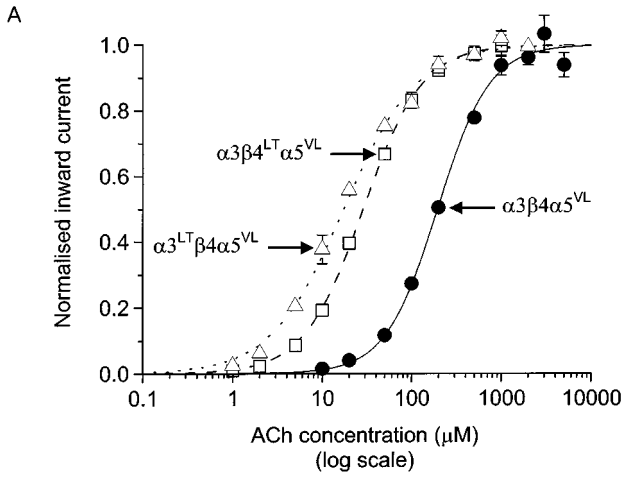

Figure 4 The magnitude of the effect of 9′T mutations in the α3 or β4 subunits on the ACh sensitivity of $\alpha3\beta4\alpha5^{VL}$ receptors is approximately equivalent. Shown are the pooled ACh concentration-response curves for oocytes injected with $\alpha3\beta4\alpha5^{VL}$ (●, $n=5$), $\alpha3\beta4^{LT}\alpha5^{VL}$ (□, $n=5$) and $\alpha3^{LT}\beta4\alpha5^{VL}$ (△, $n=4$). Current traces for these combinations are shown in Figures 1–3.

(one of which had EC_{50} and Hill slope fixed at the value measured for the pair $\alpha3\beta4^{LT}$ receptors). We found that one-component fits were consistently better than two-component fits, judging by the Akaike information criterion (see Methods). In addition, the two-component fits estimated the maximum current carried by the pair receptors at less than 1% of total current. We carried out simulations of the concentration-response data that we would expect to obtain if our oocytes did indeed express two receptor populations (see Methods). From these simulations we estimated that, with this EC_{50} separation (44 fold, see Table 1), we would detect reliably the presence of pair receptors if they contributed 10% or more of the maximum current. This therefore suggests that at least 90% of the response we measured was due to current through 'triplet' receptors containing the α5 subunit.

In α3β4α5 receptors, introducing the 9′T mutation in α3 or β4 had a similar effect, independently of the residue in the 9′ position of α5

The similarity in ACh sensitivity of receptors containing $\alpha5^{wt}$, $\alpha5^{VL}$ or $\alpha5^{VT}$ (see Table 1) showed that mutating the 9′ residue in the α5 subunit does not have a noticeable effect on the agonist sensitivity of $\alpha3\beta4\alpha5$ receptors. We next investigated whether this is true for any 9′T mutation in this 'triplet' receptor type and compared wild type $\alpha3\beta4\alpha5$ receptors with the same receptors bearing the 9′T mutation in α3 or in β4. Figure 4 shows that inserting the 9′T mutation in α3 produced approximately a 10 fold increase in agonist potency on 'triplet' receptors, as ACh EC_{50} decreased from 207 ± 25.6 to 22.7 ± 2.02 μM ($n=5$ and 4, respectively; see Table 1). The effect of mutating β4 was similar, manifesting as a 7 fold decrease in the EC_{50} for ACh (to 30.7 ± 1.63 μM; $n=5$). The magnitude of the shifts produced by mutating α3 or β4 was the same irrespective of the type of α5 subunit expressed (i.e. $\alpha5^{wt}$, $\alpha5^{VL}$ or $\alpha5^{VT}$; see Table 1).

Discussion

Incorporation of the α5 subunit in functional recombinant nAChRs

Recombinant nAChRs containing α3 and α5 were fully characterized by Lindstrom and co-workers (Gerzanich *et al*., 1998; Wang *et al*., 1996; 1998; Nelson & Lindstrom, 1999). Our data agree with their observation that α5 does not affect the EC_{50} or Hill slope of the $\alpha3\beta4$ dose-response curve to ACh, but increases apparent desensitization to high ACh concentrations (an effect not shared by β3, Groot-Kormelink *et al*., 1998; Boorman *et al*., 2000).

Additionally, our data suggest efficient assembly of $\alpha3\beta4\alpha5$ receptors in oocytes (Wang *et al*., 1996), because of the consistent reduction in ACh sensitivity observed when α5 was expressed with $\alpha3^{LT}\beta4$ or $\alpha3\beta4^{LT}$. In these mutant nAChRs, addition of α5 increased the ACh EC_{50} by 4 fold ($a3^{LT}\beta4$) or 40 fold ($\alpha3\beta4^{LT}$). Our interpretation assumes that 9′ mutations do not affect subunit assembly in nAChRs: indeed such an effect of 9′ mutations has never been reported. Furthermore, α5 incorporation in receptors mutated in α3 or β4 was clear regardless of whether α5 itself was expressed as $\alpha5^{wt}$, $\alpha5^{VL}$ or $\alpha5^{VT}$. The magnitude of the EC_{50} shift was much larger if the 9′T mutation was in β4 (40 fold vs approximately 4 fold for $\alpha3^{LT}$ nAChRs). A plausible explanation for this disparity is that α5 replaces a β4 subunit in the receptor. If that is the case and if the mutation is carried by β4, α5 would affect ACh EC_{50} not just because of its incorporation, but also because it decreases the number of copies of the mutation. Wang *et al*. (1996) first suggested that α5 replaces a β subunit. This is reasonable, because α5 differs from a typical α in the putative agonist binding domain sequences and may not be able to play the role of an α subunit. This would also be in agreement with the consistent increase in single-channel conductance produced by α5 incorporation (Ramirez-Latorre *et al*., 1996; Sivilotti *et al*., 1997; Nelson & Lindstrom, 1999). If α5 replaces a β subunit, it would increase the net negative charge of the extracellular ring of charges in position 20′ of TM2 in the nAChR pore (replacing a lysine by a glutamate): this would be expected to increase single-channel conductance in a nAChR (Imoto *et al*., 1988). Net charge would not change if α5 replaced another α subunit.

9′ mutations in α5 do not affect the agonist sensitivity of α3β4α5 receptors

Mutating the 9′ valine in the TM2 domain of α5 to a hydrophilic threonine did not change the agonist sensitivity of $\alpha3\beta4\alpha5$ receptors. This is in contrast with other nicotinic-type receptors, where such mutations consistently increase agonist potency. Mutations of 9′ to threonine or serine are thought to act on channel gating by destabilizing the closed state and/or by reducing desensitization (possibly making the desensitized state of the channel conducting; Revah *et al*., 1991; Labarca *et al*., 1995; Filatov & White, 1995). These two microscopic effects should have opposite effects on the receptor macroscopic EC_{50}. On the one hand, destabilization of the closed state increases agonist efficacy and should therefore decrease agonist EC_{50}. Whether such decrease will be detectable will depend on the magnitude of the microscopic effect of the mutation and on the actual value of agonist efficacy in the wild type receptor (Colquhoun,

1998). On the other hand, a decrease in the extent of desensitization should have the contrary effect of increasing the observed agonist EC_{50}. This is because desensitized states (by definition long-lived) contribute high affinity states to the receptor activation mechanism and therefore reduce EC_{50} values in functional assays and apparent affinity constants in binding assays. The balance between these two contrasting effects of the mutation must tilt towards the latter if a receptor has particularly fast and extensive desensitization under the recording conditions used. This is clearly the case for the $\alpha3\beta4\alpha5$ receptor (see Figure 1), in which responses to high ACh concentrations sag much more quickly and extensively that those of $\alpha3\beta4\beta3$ receptors. It seems therefore likely that we cannot detect an EC_{50} change upon V9′T mutation of α5 because the gating effect of the mutation is counteracted by its effect on desensitization. The paucity of quantitative data on the microscopic activation and desensitization mechanisms of neuronal nAChR makes it difficult to make this more than a qualitatively plausible explanation. A limitation in the sensitivity of our technique comes from the *Xenopus* oocyte expression system. On one hand, oocytes are a good choice for studying complex subunit combinations, such as our 'triplets', because they allow excellent cell-to-cell control of the constructs effectively injected. Nevertheless, the dimensions of the oocyte limit the effective rate of agonist application achievable: this is a considerable disadvantage when desensitization is fast and extensive. Extending our work to transfected mammalian cell lines may achieve a better discrimination of the mutation effects on desensitization vs gating, especially since whole-cell recording also allows a better control of internal calcium concentrations, which are known to affect desensitization processes (Khiroug *et al*., 1998). An additional strategy that may help in this will be to compare the effect of different agonists on the different receptor combinations.

It is interesting that L9′T mutation of α3 or β4 in $\alpha3\beta4\alpha5$ receptors did reduce ACh EC_{50}, but only by 10 and 6 fold, respectively. The corresponding mutations produced 17–20 fold decreases in the EC_{50} of $\alpha3\beta4\beta3$ receptors. Even in the presence of a mutated α3 or β4 subunit, V9′T mutation in α5 still had no effect on agonist potency. This could be due to saturation in the effect of 9′ hydrophilic mutations at high numbers of mutation copies, as seen for $GABA_A$ receptors (Chang & Weiss, 1999) and $\alpha3\beta4\beta3$ nAChRs (Boorman *et al*., 2000). Conversely, the lack of effect of α5 V9′T may again be due to the cancelling out of the effects of the mutation on desensitization and on gating, even though additional threonine mutations are present on the other receptor subunits. For this to be true, the 9′ residue of α5 would have to have a much bigger impact on desensitization than the same residue in the other subunits. Assessing which is the correct explanation will require experimental evidence from receptors expressed in a cell line plus an adequate model for desensitization.

Stoichiometry of α3β4α5 receptors

We recently showed that $\alpha3\beta4\beta3$ receptors have a stoichiometry of 2 : 2 : 1 (Boorman *et al*., 2000): this conclusion was reached because introducing the 9′T mutation in the different subunits produced a consistent and progressive increase in agonist potency. This was in agreement with a progressive increase in the effects of the mutation in parallel with the number of copies of the mutation incorporated, as is the case for muscle nAChRs (Labarca *et al*., 1995) and, in our hands, for $\alpha3\beta4$ receptors. Because of the similarity between β3 and α5, we suggested that all triplet receptors containing these subunits may contain two copies of the typical α subunit and two copies of the typical β subunit together with only one copy of β3 or α5.

The simplest form of this approach relies on the mutation having a simple effect that is consistent and relatively independent of which subunit carries the mutation. Clearly, both assumptions failed in the case of $\alpha3\beta4\alpha5$ nAChRs. Can we nevertheless draw any conclusions on the stoichiometry of $\alpha3\beta4\alpha5$ receptors? Subunit stoichiometry in a pentameric receptor containing three different subunits can only be 2 : 2 : 1 or 3 : 1 : 1. A 2 : 2 : 1 stoichiometry is still the one that best accords with the data, particularly with the equivalence of mutating α3 or β4 and with α5 taking the place of a β subunit.

This stoichiometry could be checked (despite the lack of an independent and equivalent effect of the mutation) by expressing mixtures of wild type and mutant for each of the subunits and counting the different components of the dose response curve (Labarca *et al*., 1995; Chang *et al*., 1996). This is only possible if each mutation produces a substantial shift in EC_{50}, sufficient to allow the unequivocal determination of up to three components in the interval of the shift. In our case, the 6–10 fold shifts observed when β4 or α3 are mutated are too small: maybe a different hydrophilic substitution (perhaps to serine) would produce a larger shift and make this approach possible.

In conclusion, our data indicate that α5 is incorporated in oocyte-expressed $\alpha3\beta4\alpha5$ nAChRs and contributes to this pentameric nAChR only one copy, which takes the place of a β subunit. Furthermore, α5 and its 9′ TM2 residue had a profound effect on receptor desensitization, possibly greater than the effect of other subunits.

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