Functional contributions of α5 subunit to neuronal acetylcholine receptor channels

J. Ramirez-Latorre, C. R. Yu, X. Qu, F. Perin*, A. Karlin† & L. Role‡

Center for Neurobiology and Behavior, Department of Anatomy & Cell Biology, *Ecole Normale Supérieure, †Center for Molecular Recognition, College of Physicians and Surgeons of Columbia University, P.I. Annex 807, 722 168th Street, New York, New York 10032, USA

Ligand-gated ion channels are multi-subunit complexes where each subunit-type is encoded by several related genes. Heterologous expression of any one of the neuronal nicotinic acetylcholine receptors (nAChR) α-type subunits, either alone or with any β-type subunit, typically yields functional nAChR channels1-4. A striking exception is the nAChR α5 subunit: although apparently complexed with β2 and β4 nAChR subunits in neurons5-8, and expressed in a subset of neurons within the central and peripheral nervous systems9-11, heterologous expression of α5, either alone or with any β-type subunit has failed to yield functional channels12-14. We demonstrate here that α5 does participate in nAChRss expressed in heterologous systems and in primary neurons, and that α5 contributes to the lining of functionually unique nAChR channels, but only if coexpressed with both another α- and β-type subunit. Furthermore, channels containing the α5 subunit are potently activated and desensitized by nanomolar concentrations of nicotine.

The neuronal nicotinic receptors are ligand-gated, cation-selective channels, made of combinations of at least two subunit types5,12-14. Three of the nAChR subunit gene products, α5, α6 and β3, have failed to yield agonist-gated currents when stimulated by β-amyloid to produce neurotoxic agents. Microglia appear to be an important mediator of neuronal death in degenerative diseases of the brain.
coexpressed in oocytes with any one of the other α or β genes\(^a\) (M. Ballivet, personal communication). The apparent lack of function in these studies has been particularly puzzling because α5 messenger RNA is expressed in specific regions of mammalian central nervous system (CNS) and communoprecipitated with β2 and β4 (ref. 8) from avian nerve tissue extracts.

Injection of chick α5 mRNA alone or coinjection of α5 with either α4 or β2 yielded no detectable macroscopic currents in response to acetylcholine (ACh) or nicotine (> 1 mM; Fig. 1a). In contrast, coinjection of all three mRNA species yielded ACh-induced macroscopic currents. When similar quantities of mRNA were injected, the maximum current obtained from α5 + α4 + β2 was typically greater than that obtained from α4 + β2 (Fig. 1b). Repeated application of 100 μM ACh evokes currents of consistent amplitude in oocytes coexpressing either α4 + β2 or α5 + α4 + β2. The same test protocol using nicotine as the agonist reveals a greatly enhanced inactivation rate of the α5 + α4 + β2 currents as compared with those obtained by injection of α4 + β2 (Fig. 1c).

After coexpression of α4 + β2, the dependence of current on agonist concentration was fitted by the Hill equation with a half-maximal effective concentration (EC\(_{50}\)) of 0.8 μM both for ACh and for nicotine. In contrast, after coexpression of α5 + α4 + β2, the dose–response data were fitted best by the sum of two Hill equations, one with a low EC\(_{50}\), corresponding to that of α4 + β2, and one with an EC\(_{50}\) roughly 125-fold greater (Fig. 2). For ACh, the two EC\(_{50}\) values were 0.8 μM and 100 μM. The EC\(_{50}\) for nicotine was increased roughly 15-fold by the coexpression of α5 + α4 + β2.

In both outside-out and cell-attached patches, channels expressed after the injection of α4 + β2 mRNA had a conductance of 24 ± 3 pS (n = 10; Fig. 3a, c), in agreement with previous reports\(^a, b, c\). Coinjection of α5 + α4 + β2 yielded an additional conductance class of 44 ± 4 pS (n = 11). The larger conductance class was detected with ratios of α5 : α4 : β2 mRNA as low as 1 : 1 : 1, and its relative abundance increased with the injection of increased amounts of α5 relative to α4 and β2 mRNA (Fig. 3b, d). Because the 44 pS channel was never detected in the absence of α5, this channel is dependent on the coinjection of α5 with α4 and β2. Although the conductances of nAChR composed of α4 + β2 and α5 + α4 + β2 were identical whether gated by nicotine or by ACh (Fig. 3c, d), the opening probability and the rate of desensitization of nicotine-gated α5 + α4 + β2 channels were greater than those of α4 + β2, even at concentrations of nicotine < 1 μM (Fig. 3c, d, right panel).

Examination of native nAChR confirms that α5 may contribute to the biophysical and pharmacological profile of nACh channels in vivo, where α5 is predominantly expressed in limbic and

**FIG. 1** Coexpression of α5 with α4 + β2 yields ACh-evoked macroscopic currents larger than those with α4 + β2 alone. a, ACh-evoked currents in oocytes and injection of mRNA as follows: (proceeding clockwise, beginning with upper left current) α4 : β2 (1 ng : 1 ng), α5 : β2 (3 ng : 3 ng), α5 : α4 (3 ng : 3 ng) and α5 : β2 (5 ng : 1 ng : 1 ng). Robust currents are seen following coexpression of α4 + β2 or α5 + α4 + β2, whereas no current is detected with application of ACh (100 μM) in oocytes previously injected with either α5 + β2 or α5 + α4 + β4. b, Data summary of ACh-evoked macroscopic current amplitudes in oocytes previously injected with varying amounts of α4 + β2 (open bars) versus α5 + α4 + β2 (black bars) mRNA. The numbers along the ordinate axis indicate the amount (in ng) of α5, α4 and β2 RNA injected (for example, 3.0/0.6/3.0 indicates injection of 3 ng α5, 0.6 ng α4 and 3 ng β2). c, Nicotine evoked currents in oocytes and injection of RNA as follows: upper panel, α4 : β2 (1 ng : 1 ng); lower panel, α5 : α4 + β2 (10 ng : 1 ng : 1 ng). Repeated nicotine application (100 μM, 5-s duration, 5-min intervals) evokes currents of consistent amplitude in oocytes injected with α4/β2 complementary RNA (upper panel). The same application causes a marked desensitization of the currents evoked in α5+α4+β2 injected oocytes (lower panel).

**METHODS** Physiology: Macroscopic currents were recorded with a 500 Gene-Clamp voltage-clamp (Axon Instruments) with an active ground. The voltage, current and active ground electrodes were filled with 3 M KCl such that voltage and current recording electrodes were ~1–5 MΩ.  

**V\(_{\text{clamp}}\) = –60 mV. Extracellular recording solution (mM): 82.5 NaCl, 2 KCl, 1 MgCl\(_2\), 10 HEPES, 1 μM atropine, pH 7.5. ACh concentration, 10 μM for α4/β2 and 1,000 μM (10 times the EC\(_{50}\) for both) for α5+α4+β2. Oocyte and RNA preparation: Oocytes were obtained following incubation of Xenopus oocytes in 2 mg ml\(^{-1}\) collagenase (Type I, Sigma) in ND96 (Specialty Media). The oocytes were incubated at room temperature for 3–4 h, and the connective tissue removed. The eggs were then washed 4 times in Barth's media, transferred to L-15, incubated overnight at 18 °C, and the RNA injected the next day. RNA was prepared from cDNAs in P7G19, a vector which contains 5' and 3' untranslated Xenopus globin regions. This vector was linearized and used as a template for run-off transcription, using the T7 promoter. The RNA used for transcription was 0.2 μg ml\(^{-1}\), and the volume injected was 20 nl per oocyte.
autonomic regions\textsuperscript{4,5}. Subunit-specific antisense oligonucleotides were used to functionally ‘delete’ $\alpha 5$ from sympathetic neurons, where the nAChR channel profile and contribution of other nAChR subunits have been characterized in previous studies\textsuperscript{10,15}. Treatment with control oligonucleotides (see legend to Fig. 3) has no effect on the three nAChR subtypes normally expressed (\textalpha 15, 33 and 53 pS). Treatment with $\alpha 5$-specific antisense oligonucleotides and assay by single-channel recordings, revealed a selective deletion of the largest (53 pS) conductance channel and no alteration in expression of either the 15 or the 33 pS nAChR subtypes (Fig. 3c, f). Furthermore, assay of the agonist dependence of the residual current in these ‘$\alpha 5$-minus’ neurons reveals significant decreases in the EC\textsubscript{50} values (C.R.Y. and L.R., manuscript in preparation). Thus the properties of nAChRs deleted by $\alpha 5$-antisense treatment are reminiscent of heterologously expressed nAChRs that include $\alpha 5$.

Although these results imply the participation of $\alpha 5$ in native and heterologously expressed nAChR complexes, alternative possibilities include an influence of $\alpha 5$ on the assembly of nAChRs with new $\beta$ stoichiometries. We sought direct proof for participation of the $\alpha 5$ subunit per se in nAChR channel complexes. If the $\alpha 5$ subunit participates directly in a functional complex with $\beta 4$ and $\beta 2$, then a region of the $\alpha 5$ sequence is likely to contribute to the ion-conduction pathway. To test this, we mutated individual residues in the M2-membrane-spanning segment (Fig. 4b, inset) of the $\alpha 5$ sequence to cysteine and expressed the mutant $\alpha 5$ mRNA together with $\beta 4$ and $\beta 2$ mRNA. The residues mutated align with those previously shown to be exposed in the channel of the mouse muscle AChR\textsuperscript{10}. In mouse muscle AChR, these residues, when mutated to cysteine, reacted with charged, hydrophilic, sulphhydryl-specific methanethiosulphonate (MTS) derivatives, and this reaction inhibited the ACh-induced current. We injected oocytes with wild-type or mutant $\alpha 5$ and $\beta 4 + \beta 2$, where the mutated $\alpha 5$ sequences were altered as follows: S240C, L246C, V247C, S248C, L254C and E257C (Fig. 4b). We tested the susceptibility of these mutants to the positively charged MTS derivative, MTS-ethylthiymethylammonium\textsuperscript{16} (MTSET; Fig. 4a).

MTSET (1 mM for 2 min) irreversibly inhibited ACh-induced currents when the nAChRs contained the mutants, $\alpha 5$-S240C, $\alpha 5$-S248C, $\alpha 5$-L254C and $\alpha 5$-E257C. The extent of irreversible inhibition of ACh-evoked currents (100 $\mu$m ACh) averaged 34%, 21%, 32%, and 28%, respectively (Fig. 4b). MTSET did not irreversibly alter ACh-evoked currents when nAChR complexes contained wild-type $\alpha 5$ or the mutants, L246C or V247C; that is, as in mouse muscle AChR, only certain of the residues of M2 are accessible to MTSET in the channel (Fig. 4b). The inhibition of cysteine-substituted mutants of $\alpha 5$ demonstrates that $\alpha 5$ is part of a functional receptor complex and that the $\alpha 5$-M2 segment contributes to the lining of the channel.

The proposed contributions of $\alpha 5$ to ligand-binding and nAChR conductance are compatible with previous studies. For example, in muscle nicotinic receptor, the aligned residues in $\alpha$, $\beta 1$, $\beta 2$, and $\delta$ at P2 (Fig. 4b) are major determinants of cationic selectivity and of ionic conductance\textsuperscript{11}. The conductance is determined by the volume and hydrophobicity of the substituted side chain\textsuperscript{22}. The participation of $\alpha 5$ in a pentameric complex would imply a change of at least one $\alpha 5$ subunit for either an $\alpha 4$ subunit or a $\beta 2$ subunit. In either case, this would yield a substitution of a Thr by a Ser (P2), with a resultant decrease in side-chain volume. Also, an increase in the negative charges at P20 (the ‘outer ring’ of charges) can alter conductance\textsuperscript{10}. The $\alpha 5$ subunit contains Glu Glu at P19 and P20, compared to Thr Gly in $\alpha 4$ and Ser Lys in $\beta 2$ (Fig. 4b). Together these three differences might account for the increase in conductance. The reaction of E257C at P19 with MTSET shows that this residue is exposed in the channel.

Six residues in the Torpedo $\alpha$-subunit have been labelled by ACh-binding-site affinity reactions (see ref. 21 for review), and these residues are highly conserved among $\alpha$-subunits. One of these residues is $\zeta$ Tyr 190\textsuperscript{22-23}, the mutation of which has profound effects on ACh binding and gating\textsuperscript{24-26}. Only in $\alpha 5$, where it is replaced with an Asp, is Tyr 190 not conserved. This alteration could explain the 125-fold increase in EC\textsubscript{50} of a population of receptors, assuming that the residues around Asp190 in $\alpha 5$, by analogy with $\alpha 1$, participate directly in the binding of ACh. Another possibility is that another region of $\alpha 5$, by analogy with

FIG. 2. The coinfusion of $\alpha 5$ with $\alpha 4 + \beta 2$ cRNA into Xenopus oocytes yields a second, lower apparent affinity component to the ACh dose–response curve. Concentration–dependence of ACh-evoked macroscopic currents in oocytes previously injected with $\alpha 4 + \beta 2$ (open circles; 0.5 $\mu$m $\alpha 5$ and 0.5 $\mu$m $\beta 2$) compared with those injected with $\alpha 5 + \beta 4 + \beta 2$ (filled circles; 5 $\mu$m $\alpha 5$, 0.5 $\mu$m $\alpha 4$, 0.5 $\mu$m $\beta 2$). ACh-evoked responses with coinjection of $\alpha 4 + \beta 2$ RNA are fitted by the function $f(x) = (1 + (K_x/x^{1})^{-1})^{-1}$ with $K_x = 0.8 \pm 0.07 \mu$m, $n_x = 1.34 \pm 0.13$, whereas $K_x$ indicates a single EC\textsubscript{50} = 0.8 $\mu$m. In contrast, coinjection of $\alpha 5$ with $\alpha 4 + \beta 2$ yields a biphasic dose–response curve fitted by the function $g(x) = (A - 1)\{1 + (K_x/x^{1})^{-1}\}^{-1} + A\{1 + (K_x/x^{1})^{-1}\}^{-1}$ Using a self-consistent iterative procedure to fit these parameters, we obtain $A = 0.80 \pm 0.01, K_x = 1.014 \pm 3 \mu$m, $n_x = 1.43 \pm 0.1$. The third line represents the calculated contribution of the low-affinity curve alone, $h(x) = (1 + (K_x/x^{1})^{-1})^{-1} = K_x = EC_{50}$. This analysis indicates that the high-affinity component of the biphasic curve represents the $\alpha 4 + \beta 2$ contribution to the current, and the value of $A$ indicates that~20% of the current is carried by $\alpha 4 + \beta 2$ (at this ratio of $\alpha 5$, $\alpha 4$ and $\beta 2$ subunit RNAs 10 : 1 : 1). The shift between the high- and low-affinity EC\textsubscript{50} is~125 fold. Increasing the amount of $\alpha 5$ RNA relative to the $\alpha 4$ and $\beta 2$ (for example to $\alpha 5$ (10 ng) $\times \alpha 4$ (0.5 ng) $\beta 2$ (0.5 ng)) does not further decrease the contribution of the $\alpha 4 + \beta 2$ current, such that a $\alpha 5$ : $\alpha 4 + \beta 2$ ratio of 0.05 : 0.5 results in EC\textsubscript{50} = 0.8 $\mu$m, EC\textsubscript{50} = 0, $A = 0$ (n = 9). A ratio of 5 : 1 produces EC\textsubscript{50} = 0.75 $\mu$m, EC\textsubscript{50} = 96.4 $\mu$m, $A = 0.27$ (n = 3). A ratio of 10 : 1 : 1 results in EC\textsubscript{50} = 0.835 $\mu$m, EC\textsubscript{50} = 101.4 $\mu$m, $A = 0.2$ (n = 6) and a ratio of 20 : 1 : 1 produces EC\textsubscript{50} = 0.77 $\mu$m, EC\textsubscript{50} = 100 $\mu$m and $A = 0.2$ (n = 3). The analysis using the Hill equation assumes infinite cooperativity, and in spite of its widespread use, is valid only in the limiting case. Using a more realistic cooperative binding scheme, one obtains for the case of two binding sites with an energy of interaction $\Delta G_w$, the grand canonical partition function, $z = 1 + \exp(-\Delta G_w)/kT$, where $x = e^\Delta G/wK$ and $y = \exp(-\Delta G/wK)$ (c is the ligand concentration, $K$ is Boltzmann constant and $T$ is the temperature). For the occupancy function $\theta = (k_x(x + k_x)^{1 - 1})/(1 + x + k_x^2)$, using this function we obtain, $K_x = 2.5 \mu$m, $K_y = 280 \mu$m, $x = 10 (w_{\alpha 5} = 1.347 \text{ cal mol}^{-1} \text{ at 300 K})$. The fit of these functions to the $\alpha 4 + \beta 2$ and $\alpha 5$/$\beta 2$ dose–response curves is shown as dotted lines in the figure. The equivalent Hill coefficient is related to fluctuations in the extent of binding, and is obtained by $n = 2 + 1/y_{\alpha 5}/y_{\beta 2}$, which is in this case $n = 2y_{\alpha 5}/(1 + y_{\alpha 5})$, and gives $n = 1.5$ for both the low- and high-affinity curves. Because of the size of the oocyte, there is considerable spread of the activation of receptors, which distorts the measurement of the macroscopic current kinetics. This measurement is further complicated by inaccessibility of up to 30% of receptors because of membrane infoldings and the presence of the vitelline membrane (unpublished observations).
FIG. 3 Coexpression of α5 with α4 + β2 yields a new nAChR channel subtype of higher conductance. ACh-gated single-channel currents in outside-out patches from oocytes previously injected with α4 + β2 (a) compared with those obtained following the coexpression of α5 + α4 + β2 (b) RNA injection. a, Left, single channels recorded from an outside-out patch in an oocyte injected with α4 (1 ng) β2 (1 ng) RNA. Membrane potential = −100 mV. ACh concentration, 1 μM. Middle, a single Gaussian fit yields m1 = 2.36 ± 0.4 pA. Right, the single-channel conductance from linear regression of the IV plot is 23.96 ± 3 pS. b, Left, typical single-channel currents from an outside-out patch following injection of α5 (3 ng) α4 (1 ng) β2 (1 ng) RNA in oocyte. Membrane potential = −100 mV. ACh concentration, 100 μM. Middle, the amplitude histogram of the recorded currents is fit by the sum of two gaussian curves with m1 = 2.8 ± 0.7, m2 = 4.3 ± 0.9. Increasing the amount of α5 mRNA relative to α4 + β2 decreases the number of events within the m1 Gaussian and increases the number of events described by m2. c, Right, the single channel conductance of the higher amplitude class is 44 ± 3.6 pS as estimated by linear regression of the IV plot. Coexpression of α5 with α4 + β2 is without effect on the conductance of the smaller channel (25 ± 3.5 pS). The linear regressions to assess single-channel conductance were constrained to pass through the origin (a, b, c, Left, single channels recorded from an oocyte injected with α4 (1 ng) β2 (1 ng) RNA. Membrane potential, −100 mV. Nicotine concentration, 0.2 μM or 1 μM, as indicated. Middle, amplitude histogram of the recorded events is fit by a single Gaussian (excluding zero events) with mean and s.d. m1 = 2.3 ± 0.8 pA. A slope conductance measurement for this patch, using events at −60, −80 and −100 mV yields γ = 21 ± 4 pS. d, Left, typical single-channel currents from an outside-out patch from an oocyte injected with α5 (1 ng) α4 (0.1 ng) β2 (0.1 ng) RNA. Membrane potential, −100 mV. Nicotine concentration 0.2 μM or 1 μM as indicated. Middle, amplitude histogram, fit by two Gaussians (excluding zero events) with means and s.d., m1 = 2.1 ± 1.6 pA and m2 = 3.9 ± 1.1 pA. Slope conductance γ = 45 ± 6 pS (using events at −60, −80 and −100 mV). e, Typical single-channel recordings from chick sympathetic neurons (ED11) reveals three conductance classes (γ1 = 15 pS, γ2 = 33 pS, γ3 = 53 pS). f, Treatment with α5 antisense deletes the high conductance class (γ3 = 53 pS) and is without effect on lower conductance classes (γ1 = 18 pS, γ2 = 30 pS). Neurons were dispersed from ED11 lumbar sympathetic ganglia, maintained in vitro and treated with antisense as described previously27. A three-base mismatch α5-antisense sequence served as control. Single-channel recordings were obtained using standard techniques28 with an Axon 200A patch-clamp (Axon Instruments). Solutions (in mM): Solution 1 (bath): 115 NaCl, 2 KCl, 1 CaCl2, 1 MgCl2, 10 HEPES, pH 7.5; solution 2 (pipette): 115 KCl, 0.2 EGTA, 10 HEPES, pH 7.5.
FIG. 4 Cysteine substitution mutagenesis within the α5-M2 sequence demonstrates that α5 participates in functional nACHR complexes and that the α5-M2 segment contributes to the lacing of the channel. The cysteine-reactive reagent MTSET irreversibly reduces the magnitude of ACh-evoked currents in oocytes previously injected with α5 + α4 + β2 RNA only when the α5 sequence has been mutated to include cysteine residues at specific sites within α5-M2. a, Comparison of the effect of MTSET on ACh-evoked currents in oocytes coinjected with WT α5 + α4 + β2 RNA (5 nM/0.5 ng/0.5 ng; top traces) versus those coinjected with α5 mutated, so that a cysteine residue is substituted at α5SE257, α5SE257 (+4) β2 (α5SE257C; 5 nM/4.5 ng/0.5 ng; bottom traces). The effects of MTSET are tested as follows: (1) determine average peak response to 2–3 sequential applications of ACh (100 μM; 5 min inter-test interval); (2) perfuse with control oocyte recording media (5 min); (3) perfuse with oocyte recording media with MTSET (1 mM; 2 min, indicated by open bar); (4) repeat control perfusion as in (2); (5) repeat ACh applications as in (1). The records shown in are paired first or second responses to ACh before and after MTSET treatment, respectively. b, Summary of experiments using cysteine substitution in α5-M2 to test participation of α5-M2 in the channel lacing. The inhibition of ACh-evoked currents by MTSET was evaluated as described in a. Box shows the aligned TM2 regions for mouse α1 and chick α4, α2 and α5. Note that α5 sequence numbering is used (for example, α5S240 aligns with α1N244). Oocytes injected with the WT and mutant subunit cRNAs indicated on the ordinate. Six sites within α5-M2 (in bold) were mutated to a cysteine, with the sites chosen to correspond to those in x1 (muscle type, muscle-type γ and δ subunits, participates in the binding of ACh). A third possibility is that the change in EC50 is an indirect effect of the changed and perhaps varying subunit composition.

Ultimately one must attempt to relate the characteristics of heterologously expressed nACHRs with those of native nACHRs in the central and peripheral nervous system. In this regard, coexpression of each of the known α- and β-subunits has failed to produce channels that resemble the higher conductance (40–60 pS) nACHR subtypes expressed in sympathetic, ciliary, habenular, interpeduncular or hippocampal neurons of rat or chick (see ref. 5 for review). The inclusion of α5 with other α- and β-subunits may be important in the formation of these high-conductance nACHRs in these regions, where α5 is expressed. Perhaps most striking with regards to the potential physiological relevance of α5-containing AChRs is the potent activation of α5-containing AChRs by nicotine and the differential desensitization of these channels by submicromolar concentration of this drug (Figs 1c and 3b, c).

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