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Functional characterization of a mutated chicken α_7 nicotinic acetylcholine receptor subunit with a leucine residue inserted in transmembrane domain 2

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1 Site-directed mutagenesis was used to create an altered form of the chicken α_7 nicotinic acetylcholine (ACh) receptor subunit (α_7x61) in which a leucine residue was inserted between residues Leu9′ and Ser10′ in transmembrane domain 2. The properties of α_7x61 receptors are distinct from those of the wild-type receptor.

2 Oocytes expressing wild-type α_7 receptors responded to 10 μM nicotine with rapid inward currents that desensitized with a time-constant of 710 ± 409 ms (mean $\pm$ s.e.mean, $n=5$). However in α_7x61 receptors 10 μM nicotine resulted in slower onset inward currents that desensitized with a time-constant of 5684 ± 3403 ms (mean $\pm$ s.e.mean, $n=4$). No significant difference in the apparent affinity of nicotine or acetylcholine between mutant and wild-type receptors was observed. Dihydro-β-erythroidine (DHβE) acted as an antagonist on both receptors.

3 Molecular modelling of the α_7x61 receptor channel pore formed by a bundle of M2 α-helices suggested that three of the channel lining residues would be altered by the leucine insertion i.e.; Ser10′ would be replaced by the leucine insertion, Val13′ and Phe14′ would be replaced, by Thr and Val, respectively.

4 When present in the LEV-1 nicotinic ACh receptor subunit from *Caenorhabditis elegans* the same alteration conferred resistance to levamisole anthelmintic drug. Levamisole blocked responses to nicotine of wild-type and α_7x61 receptors. However, block was more dependent on membrane potential for the α_7x61 receptors.

5 We conclude that the leucine insertion in transmembrane domain 2 has the unusual effect of slowing desensitization without altering apparent agonist affinity.

Keywords: Nicotinic acetylcholine receptor (nicotinic AChR); α_7 subunit; channel mutation; desensitization; agonist affinity; levamisole

Introduction

Nicotinic acetylcholine (ACh) receptors are the most extensively studied members of the cys-loop family of ionotropic neurotransmitter receptors (Unwin, 1993a,b; Karlin & Akabas, 1995; Galzi & Changeux, 1995). The nicotinic ACh receptors of vertebrate skeletal muscle and the electroplax organ of electric fish (a modified muscle) are composed of five subunits ($\alpha_2\beta\gamma\delta$) arranged around an aqueous ion pore (Unwin, 1989; 1993a,b). The α-subunits include a pair of vicinal cysteine residues (*Torpedo* Cys192 and Cys193) which are located within 10 Å of the ACh binding site (Karlin, 1969; Kao *et al*., 1984). Less is known of the organization of neuronal nicotinic ACh receptors; however, there is evidence that they are also pentamers (Cooper *et al*., 1991). Neuronal nicotinic ACh receptor subunits are classified as belonging to one of two types; α subunits which have vicinal cysteine residues homologous to those found in muscle α-subunits (*Torpedo* Cys192 and Cys193) and non-α (or β) subunits which lack this motif. Several isoforms ($\alpha_{2\text{-}9}$, $\beta_{2\text{-}4}$) of both subunit types have been identified. Certain neuronal α subunit isoforms, notably α_7, form functional homo-oligomers when expressed in *Xenopus* oocytes (Couturier *et al*., 1990; Bertrand *et al*., 1992a).

All nicotinic ACh receptor subunits belong to a superfamily of neurotransmitter receptor subunits which are distinguished by the presence of a dicysteine-delimited loop (cys-loop) in the N-terminal, extracellular domain. In addition each subunit has four putative membrane-spanning domains (M1–4) (Karlin & Akabas, 1995). Together the M2 regions of each of the five subunits in a receptor form the lining of the channel pore. Cryoelectron microscopy studies have suggested that M2 is formed by a kinked α helix with the narrowest point of the channel at the level of a highly conserved leucine residue, Leu9′ (Unwin, 1989; 1993a,b). Throughout this paper the numbering system of Miller (1989) and Lester (1992) is used to represent residues in M2 (see Figure 1). Substituted-cysteine accessibility method (SCAM) studies also suggest an irregular structure between Leu8′ and Ser10′ in the α subunit of the muscle receptor. Photoaffinity labelling with non-competitive blockers, site-directed mutagenesis, SCAM analysis and molecular modelling have identified many of the same residues as being channel lining (see Karlin & Akabas, 1995 for review and also Sansom *et al*., 1995; Sankararamakrishnan *et al*., 1996). Results from labelling experiments with non-competitive inhibitors and site-directed mutagenesis studies in which homologous residues in different subunits within a hetero-oligomeric receptor behave in very similar ways suggest that the arrangement of the five M2 segments around the channel pore is quasisymmetrical (Karlin & Akabas, 1995).

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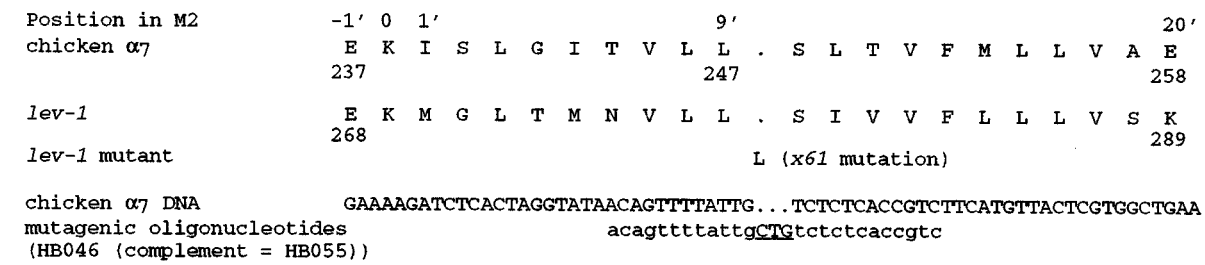

Figure 1 Mutagenesis of the chicken α_7 nicotinic ACh receptor subunit cDNA to incorporate the *lev-1 x61* mutation. The figure shows the aligned amino acid sequences of the M2 regions of the chicken α_7 nicotinic ACh receptor subunit and the *C. elegans* nicotinic ACh receptor LEV-1 subunit. The position of the leucine insertion which gives rise to the semi-dominant levamisole resistant phenotype in the *lev-1 (x61)* mutant strain is shown. This same mutation was introduced into the chicken subunit using a PCR-based mutagenesis protocol. Two complementary oligonucleotides (HB046 and HB055) representing the desired α_7 sequence were synthesized and used in PCR reactions to produce fragments of the α_7 DNA with the desired alteration. These fragments were then reunited by means of a second PCR reaction and subcloned back into the wild-type chicken α_7 subunit. The positions of amino acid residues within M2 are given, throughout this paper, by using the numbering system of Miller (1989) and Lester and co-workers (Lester, 1992; Kearney *et al*., 1996). These are shown above the chicken α_7 sequence in the figure. The absolute numbering of the amino acid residues in the chicken α_7 and LEV-1 subunits are shown below each sequence. Leu9′ is equivalent to Leu251 of the muscle α subunits.

It is clear that the highly conserved Leu9′ residue in M2 plays a pivotal role in the function of the receptor although its exact role remains controversial. The results of cyroelectron microscopy and modelling have led to models in which Leu9′ functions as the channel gate and as the point of a molecular swivel, rotation about which, leads to channel opening (Unwin, 1989; 1993a,b; Sansom, 1995). However the results of SCAM analysis (Akabas & Karlin, 1995) and a number of site-directed mutagenesis studies have led to discussion of whether or not Leu9′ acts as a gate and/or plays a role in desensitization. Molecular dynamics simulations of pore/ion/water interactions suggest that simple occlusion of the central pore by Leu9′ may not be sufficient to account for channel gating (Smith & Sansom, 1997). Substitutions of Leu9′ and at a number of other residues (e.g. Val 13′ Thr) result in alterations in apparent agonist affinity, rate of desensitization (decreased when apparent agonist affinity is increased) and alterations in the mean open time (increased when apparent agonist affinity is increased) (Revah *et al*., 1991; Bertrand *et al*., 1992b; Labarca *et al*., 1995; Filatov & White, 1995; Kearney *et al*., 1996 and see Galzi *et al*., 1996 for discussion). A number of mutations that result in congenital slow-channel myasthenic syndromes have been identified in the M2 regions of the human muscle α,β and ε subunits e.g. αVal7′(249)Phe, αThr12′(254)Ile, βVal12′(266)Met, εThr11′(264)Pro and εLeu16′(269)Phe. These mutants also show increased apparent agonist affinity, increased spontaneous opening, slower channel closing and enhanced desensitization where this has been studied (see Engel *et al*., 1996; Croxen *et al*., 1997; Milone *et al*., 1997 and references therein).

The analysis of naturally occurring mutations in cys-loop receptors is an important source of information on structure-function relationships in these proteins and on the mechanisms of the actions of chemicals and drugs upon them. Illustrations of this include mutations in human muscle acetylcholine receptor subunits which cause congenital slow-channel myasthenic syndromes (e.g. Engel *et al*., 1996; Croxen *et al*., 1997; Milone *et al*., 1997) and a mutation in a *Drosophila* γ-aminobutyric acid (GABA) subunit (RDL) which confers resistance to insecticides such as dieldrin and fipronil (ffrench-Constant *et al*., 1991; Hosie *et al*., 1997). In the nematode, *Caernorhabditis elegans*, several viable mutants, which have altered nicotinic ACh receptors have been identified (Lewis *et al*., 1980; 1987; Treinin & Chalfie, 1995; Fleming *et al*., 1996; 1997). Application of the anthelmintic drug, levamisole, to wild-type *C. elegans* results in muscle hypercontraction and eventually death. Screens for worms which are resistant to levamisole have identified 11 genes which when mutated can confer resistance to levamisole (Lewis *et al*., 1980; 1987). At least three of these genes encode nicotinic ACh receptor subunits (Fleming *et al*., 1996; 1997). The *lev-1* gene encodes a non-α subunit (LEV-1), which is believed to form part of a levamisole-sensitive nematode nicotinic ACh receptor (Fleming *et al*., 1996; 1997). Most mutant alleles of the *lev-1* gene are recessive and confer partial resistance to levamisole (presumably as a result of the loss of LEV-1 function). However, 2 rare semi-dominant alleles (*x61* and *x21*) of the *lev-1* gene result in extremely levamisole-resistant worms (Lewis *et al*., 1980). In both cases changes in the amino acid sequence of M2 have been identified. The *x61* allele results in the insertion of a leucine residue between Leu9′ and Ser10′ in M2 (Fleming *et al*., 1997). Since the insertion of an amino acid is an unusual event in such a highly conserved domain and since no such mutants had been studied in functional nicotinic ACh receptors we have used site-directed mutagenesis to introduce the same mutation into the chicken α_7 subunit.

Methods

Preparation of the α_7x61 cDNA expression plasmid

The chicken nicotinic ACh receptor α_7 subunit (Courtier *et al*., 1990) in the vector pMT3 (Swick *et al*., 1992) was a gift from Dr M. Ballivet. A 1.2 kb *Eco*RI fragment from the α_7 cDNA which extended from the nucleotides encoding residue 178 of the native protein to the polylinker in pMT3, was subcloned into the *Eco*RI site of pALTER-*Ex1* (Promega) to yield the plasmid pHB700. Complementary oligonucleotides (HB046 and HB055) based on the chicken α_7 sequence, but containing the *lev-1 x61* mutation (Figure 1), together with oligonucleotides for the SP6 and T7 promoters flanking the insert, were used in a PCR-based mutagenesis protocol (Higuchi, 1989). All PCR reactions were performed using *Pfu* polymerase (Stratagene, to ensure high fidelity amplification), primers at 0.2 μM and for 30 cycles of 94°C 45 s, 50°C 60 s, 72°C 180 s. First round reactions (1: SP6, HB046 and 2: T7, HB055) each yielded single bands of the correct size.

After gel purification the products of reactions 1 and 2 were combined and re-amplified using the SP6 and T7 oligonucleotides. This reaction generated a product of the correct size which was cut with *Eco*RI and used to replace the wild-type *Eco*RI fragment in pMT3-α_7. Plasmids carrying the mutagenized *Eco*RI fragment in the correct orientation were identified by restriction enzyme analysis. pHB761 carried the *lev-1 x61* mutation but was otherwise identical in sequence to the wild-type plasmid. DNA for microinjection was purified using Wizard minipreps (Promega) with elution in RO quality distilled water.

Preparation and nuclear injection of Xenopus *oocytes*

Mature *Xenopus laevis* females were anaesthetized by immersion in 1.5 g l^{-1} tricaine for 30–45 min (depending on body weight) before surgical removal of part of the ovary. Oocytes at stages V or VI of development were separated from the follicular cell layer, whilst leaving the innermost vitelline layer intact, by treatment with collagenase (Sigma type 1A) (2 mg ml^{-1}) in calcium-free SOS for 30 min at room temperature followed by manual removal of the follicular layer using fine forceps. The composition of SOS was (mM): NaCl 100, KCl 2.0, $CaCl_2$ 1.8, $MgCl_2$ 1.0, HEPES, 5.0; pH 7.6. The nuclei of defolliculated oocytes were each injected with 20 nl cDNA (15 ng μl^{-1}) using a digital microdispenser (Drummond 'Nanoject'). Following injection, the oocytes were incubated, at 17°C, in SOS supplemented with 50 μg ml^{-1} gentamycin sulphate, 100 u ml^{-1} penicillin, 100 μg ml^{-1} streptomycin and 2.5 mM sodium pyruvate. The medium was changed daily, and electrophysiological experiments were performed 2–5 days after cDNA injection.

Electrophysiology

For electrophysiological studies, each oocyte was secured in the experimental chamber (approximate volume = 90 μl) and perfused continually with SOS (2 ml min^{-1}) in which $CaCl_2$ was replaced by $BaCl_2$ (1.8 mM). The use of barium ions instead of calcium in the oocyte saline was to minimize the activation of calcium-dependent chloride currents (Barish, 1983). Oocytes were impaled with two 2 M KCl-filled glass microelectrodes of resistance 2–5 MΩ. Membrane currents were amplified and monitored using an Axoclamp 2A voltage-clamp amplifier (Axon Instruments) and displayed on both a Nicolet digital oscilloscope and on a dual channel chart recorder (Gould, BS-272). The data were also recorded on video tape (Panasonic NV-FS2 1) afte pulse-code modulation (Sony PCM-701ES) for further analysis (Sattelle *et al*., 1992). In all experiments, the command potential was clamped at −60 mV unless otherwise indicated. Nicotine and all test molecules were bath-applied in the perfusing medium from an aqueous stock (10 mM). Each oocyte was challenged with saline containing 10 μM nicotine for 15 s at regular intervals in order to test for stability of the amplitude of the response.

During experiments, an interval of at least 3 min (for concentrations up to 30 μM) was allowed between nicotine challenges to reduce the effects of desensitization. This was sufficient to ensure that responses to repeated applications of 10 μM nicotine did not vary in amplitude by more than 10%. Following exposure to 100 μM nicotine it was necessary to allow 6 min before the next application of drug. Repeated exposure to nicotine concentrations above 100 μM often resulted in irreversible reduction in current responses. The actions of antagonists were determined by first measuring the amplitude of the response to 10 μM nicotine, a concentration close to the EC_{50} for nicotine. After 1–2 min wash in normal saline, the oocyte was perfused in saline containing antagonist for 1 min after which the oocyte was challenged with 10 μM nicotine in the presence of antagonist. This procedure was repeated for each antagonist challenge so that any changes in amplitude of control responses to nicotine could be detected and the data subsequently normalized.

The voltage-dependence of nicotine action was determined by measuring agonist-induced currents at clamped membrane potentials between $E_m = -140$ mV and −25 mV. In the case of the voltage-dependence of levamisole action, the amplitude of the response to 10 μM nicotine was measured at a clamped membrane potential, washing the oocyte in normal saline for 1–2 min, then applying levamisole (3×10^{-5} M) for a further 1 min, after which the oocyte was challenged with saline containing both antagonist and nicotine. This was repeated at each clamped membrane potential. Oocytes were maintained at a holding potential (E_h) of −60 mV between such trials, then clamped at the test potential just before the application of nicotine. When the membrane potential was changed sufficient time was allowed for the clamp current to stabilize before applying nicotine.

Analysis of data obtained in electrophysiological experiments

To determine the time-constant of the decay of the agonist-induced currents during desensitization responses were played back from videotape and acquired digitally using pClamp 6.01 software (Axon Instruments) on a Viglen Genie, 486DX computer.

Each data point represents the mean ± one s.e.mean of n experiments performed on separate oocytes. Curves were fitted using GraphPad Prism software (GraphPad, U.S.A.) to the equation $Y = \min + ((\max\text{-}\min)/1 + 10^{(\log EC_{50}\text{-}X)n_H})$ where Y is the dependent variable, X is the concentration of the compound, max is the maximum value of Y, min is the minimum value of Y and n_H is the Hill slope. Efficacies of compounds are expressed as pEC_{50} ($= -\log_{10}EC_{50}$).

Molecular modelling and electrostatic calculations

The structure of the M2 helix bundle forming the pore-lining domain of the mutant α_7 receptor, in the open confirmation, was modelled using simulated annealing via restrained molecular dynamics as described elsewhere (Kerr *et al*., 1994; Sansom *et al*., 1995; Sankararamakrishnan *et al*., 1996). This method incorporates restraints derived from 9Å resolution cryoelectron microscopy data (Unwin, 1995), in addition to orienting pore-lining sidechains, as identified by site-directed mutagenesis studies (Lester, 1992), towards the lumen of the pore. Continuum electrostatic calculations were performed (Sankararamakrishnan *et al*., 1996) in order to estimate the electrostatic potential energy of a cation as a function of its position along the pore (z) axis.

Results

Actions of nicotine and acetylcholine on wild-type and mutant α_7x61 *nicotinic ACh receptor homo-oligomers*

We constructed a derivative of the chicken α_7 subunit in which a leucine residue was introduced into M2 between Leu9′ and Ser10′. The mutant form was named α_7x61. To investigate the functional properties of this receptor we compared its

behaviour to that of the wild-type when expressed in *Xenopus laevis* oocytes.

First we tested the ability of the mutant subunit to form functional homo-oligomers. Responses to nicotine in the concentration range 3 μM to 1 mM were seen in 40% of all oocytes injected ($n=102$) with α_7x61 or wild-type α_7 cDNA. The absence of responses in DNA-injected oocytes was attributed to failure to inject into the nucleus. No responses to 100 μM nicotine were detected in uninjected, defolliculated oocytes ($n>20$). In nearly all injected oocytes responding to nicotine, the response stabilized within 15 min of the first application of the agonist.

The application of either nicotine (Figure 2a) or acetylcholine (Figure 2b) to oocytes expressing wild-type α_7 or the α_7x61 mutant subunit, clamped at $E_m=-60$ mV, resulted in dose-dependent inward currents which desensitized during the application of the agonist.

Dose-response curves for nicotine and ACh for both wild-type and α_7x61 mutant receptors were determined (Figure 2a and b). From these data, pEC_{50} values of 4.91 ± 0.15, $n=5$ (wild-type) and 5.23 ± 0.01, $n=5$ (α_7x61 mutant) were estimated for nicotine. Similarly pEC_{50} values of 3.97 ± 0.17, $n=5$) (wild-type) and 4.19 ± 0.11 $n=5$) (α_7x61 mutant) were estimated for acetylcholine. Thus the leucine insertion produces no significant effect on affinity. However, we did observe a change in the shape of the dose-response curve in the case of nicotine. In this case the Hill slope coefficient for the wild-type was 1.34 whilst that for mutant was 1.75.

The dose-response curves for action of ACh and nicotine on the wild-type and mutant receptors (Figure 2) showed no significant differences ($P<0.01$, Student's t test, for both agonists). No difference was observed between the rates of desensitization in response of the α_7x61 mutant receptor to nicotine and ACh.

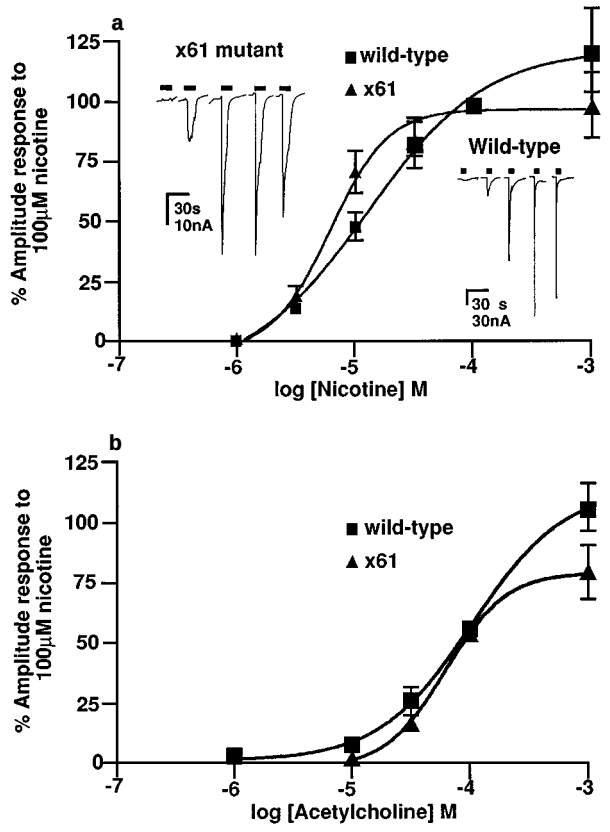

Figure 2 Dose-dependent inward currents induced by nicotine and ACh in *Xenopus* oocytes injected with cDNA encoding wild-type or *x61* mutant α_7 nicotinic ACh receptor subunits. (a) The dose-response curves for nicotine show that there is little difference in the EC_{50} value for nicotine-induced currents between the wild-type and α_7x61 receptors. The insets show the responses to increasing concentrations of nicotine recorded consecutively from the same oocytes. The concentrations are 1 μM, 3 μM, 30 μM and 100 μM (wild-type), 1 μM, 10 μM, 30 μM, 100 μM and 1 mM (*x61*). The responses are expressed as a % of the peak amplitude of the current response to 100 μM nicotine measured for each oocyte. Nicotine was applied in increasing concentrations, sufficient time being allowed between challenges to minimize the effects of desensitization. (b) The dose-response curves for ACh measured from the same oocytes as above. Plotted as for (a) above. The points at 1×10^{-3} M ACh are not significantly different (t test, $P<0.01$).

The leucine insertion in M2 changes the rate of desensitization of the mutant receptor

We compared the rate of desensitization of the wild-type and mutant receptors. The rate of desensitization of the α_7x61 mutant was considerably slower than that of the wild-type receptor (Figures 2a and 3a). In the case of the wild-type receptor the desensitizing phase of current responses to 10 μM nicotine was fitted to a negative exponential with a time-constant of 710 ± 409 ms ($n=5$). In a contrast the time-constant for the α_7x61 mutant was 5658 ± 3403 ms ($n=4$).

It is clear from Figure 3 that responses to nicotine in the mutant receptor were slower in onset than those of the wild-type receptor. The method of drug application employed was too slow to allow accurate measurement of the rate of onset.

The leucine insertion does not alter the action of dihydro-β-erythroidine (DHβE) or current-voltage properties

We tested the action of DHβE on the wild-type and mutant channels. The action of DHβE which is normally that of a

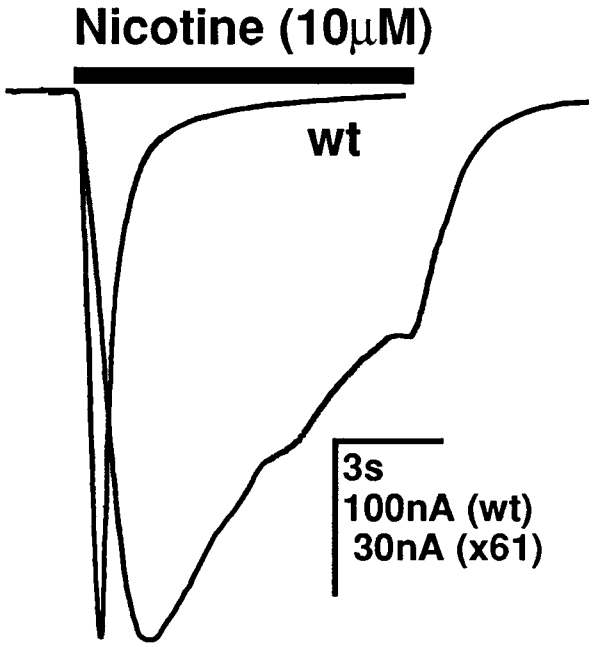

Figure 3 Comparison of responses to nicotine of wild-type and *x61* mutant receptors expressed in *Xenopus* oocytes. The response to nicotine is slower both in onset and desensitization in *x61* mutants than in wild-type α_7. Here two traces are superimposed to highlight the differences in the time course of the response. The same time scale is used in both traces.

competitive antagonist on the α_7 receptor (Bertrand *et al*., 1992a) is altered to that of an agonist by mutations in M2 which also reduce desensitization (Bertrand *et al*., 1992b). Application of 100 μM DHβE to oocytes injected with the α_7*x61* mutant cDNA did not result in any measurable membrane currents and abolished the response to nicotine (Figure 4a). DHβE therefore acts as an antagonist on both the wild-type and the α_7*x61* mutant receptor.

We also examined the current-voltage properties of the wild-type and mutant forms of the receptor (Figure 4b).

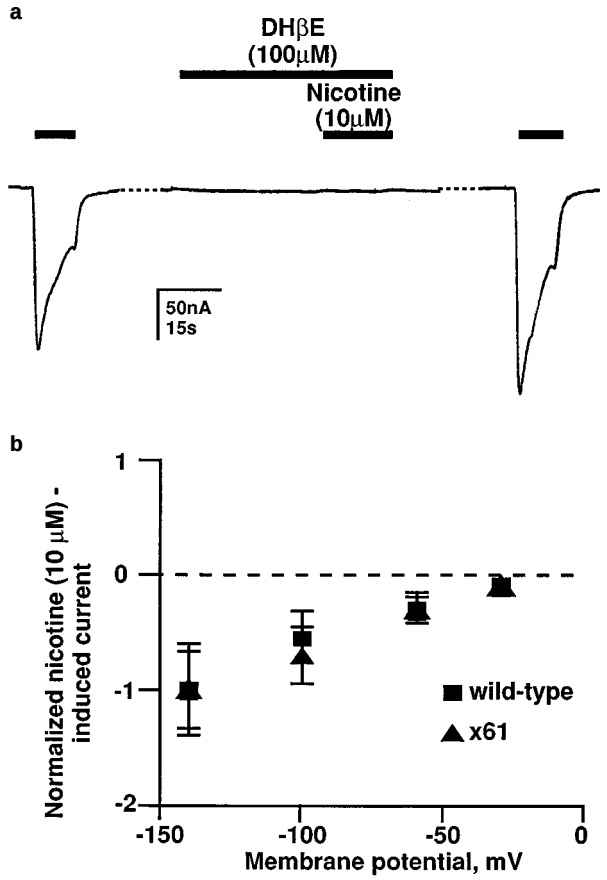

Figure 4 The leucine insertion does not alter the action of dihydro-β-erythroidine (DHβE) or current-voltage properties. (a) The response to nicotine recorded in oocytes expressing α_7*x61* mutant is reversibly blocked by the competitive antagonist, dihydro-β-erythroidine (100 μM). The oocytes were exposed to a test application of 10 μM nicotine. After 2 min wash in normal saline, the saline was exchanged for one containing 100 μM dihydro-β-erythroidine. After a further 1 min, the oocyte was then exposed to a saline containing both 10 μM nicotine and 100 μM dihydro-β-erythroidine. After 15 s exposure to this mixture of agonist and antagonist, the oocytes were washed in normal saline for 3 min, followed by another exposure to 10 μM nicotine to test for the recovery of the response. In no case did the dihydro-β-erythroidine alone result in any measurable agonist response. (b) Current-voltage relationships in wild-type and mutant α_7 nicotinic ACh receptors. The current-voltage plots for nicotine-induced responses in wild-type and *x61* mutant α_7 nicotinic ACh receptors are shown. In each case oocytes were voltage-clamped at -60 mV. Immediately (<30 s) before the application of nicotine, the clamped holding potential was changed to a test value within the range -140 mV to -25 mV and the peak amplitude of the current response to bath-applied nicotine (10 μM) measured, after which the clamped holding potential was returned to -60 mV. At least 3 min was allowed between successive nicotine applications. Responses were normalized to the maximum nicotine-induced current (-1) and plotted against membrane potential. Each point represents the mean of at least three experiments, each on a separate oocyte, and vertical lines represent s.e.mean.

Nicotine-induced currents were recorded at a range of holding potentials. Normalized responses were plotted against the clamped membrane potential. Extrapolation from measured values suggests that the reversal potentials for the two forms of the receptor are similar. Thus the leucine insertion does not produce any significant change in these properties of the channel.

Effects of the leucine insertion on levamisole actions

The insertion of the leucine residue between Leu9′ and Ser10′ in the *C. elegans* LEV-1 subunit results in strong resistance to the anthelmintic drug levamisole. Therefore the actions of levamisole were examined on both wild-type and α_7*x61* receptors expressed in *Xenopus* oocytes. Levamisole (10 μM to 1 mM) did not evoke any membrane currents when the oocytes were clamped at -60 mV but reversibly reduced the responses to nicotine in both wild-type and mutant α_7*x61* receptors (Figure 5a). We determined the value of pEC_{50} for nicotine in the presence and absence of levamisole as 4.67 ± 0.38 and 4.98 ± 0.08, respectively, for the wild-type receptor and 4.97 ± 0.16 and 4.77 ± 0.07, respectively, for the mutant receptor (no significant difference for either receptor; $P < 0.001$, Student's t test). This effect appeared to be non-competitive, in that it reduced the amplitude of responses to all concentrations of nicotine without greatly changing the EC_{50} of the agonist. Thus, its effect could be overcome completely by raising the concentration of nicotine (data not shown). The dose-dependency of the levamisole action was measured for both wild-type and mutant receptors. The α_7*x61* mutant was slightly less susceptible to the actions of levamisole. The pIC_{50} for the antagonist was estimated from the levamisole dose-inhibition curves shown in Figure 5b to be 4.33 ± 0.09 for the wild-type and 4.00 ± 0.09 for the mutant receptor (significant at $P = 0.02$, Student's t test).

We also investigated the voltage-dependence of the block by levamisole on both wild-type and α_7*x61* receptors. Whereas levamisole acted in a voltage-independent manner on the wild-type receptor its action on the α_7*x61* mutant was markedly voltage-dependent (Figure 5c).

Modelling the channel of the α_7x61 *mutant receptor*

To investigate the possible structural effects of the α_7*x61* mutation we modelled the pore lining domain, of the mutant receptor, in the open confirmation. We then compared this to a model of the α_7 wild-type receptor described previously (Sankararamakrishnan *et al*., 1996). In order to derive the model it was necessary to make some assumptions about the effects of the leucine insertion. The results described above clearly demonstrate that the mutant subunit forms channels. We assumed the N-terminal region of the channel, which is more constrained by packing interactions of adjacent M2 helices, was unchanged and hence the principal effect of the mutation would be to alter the structure on the C-terminal side of Leu9′. Based on these assumptions we were able to derive a model of the pore domain of the channel formed by the α_7*x61* mutant protein (Figure 6). The most substantial difference between the predicted structures of the mutant and wild-type receptors is that the pore lining residues, Ser10′, Val13′, Phe14′ are changed to Leu, Thr and Val, respectively, in the mutant (Figure 6a). Thus at position 10′ a polar residue is replaced by a non-polar one, whilst at 13′ a non-polar residue is replaced by a polar residue. Therefore the overall level of the polarity of this region of the pore-lining is more or less conserved. At position 14′ the size of

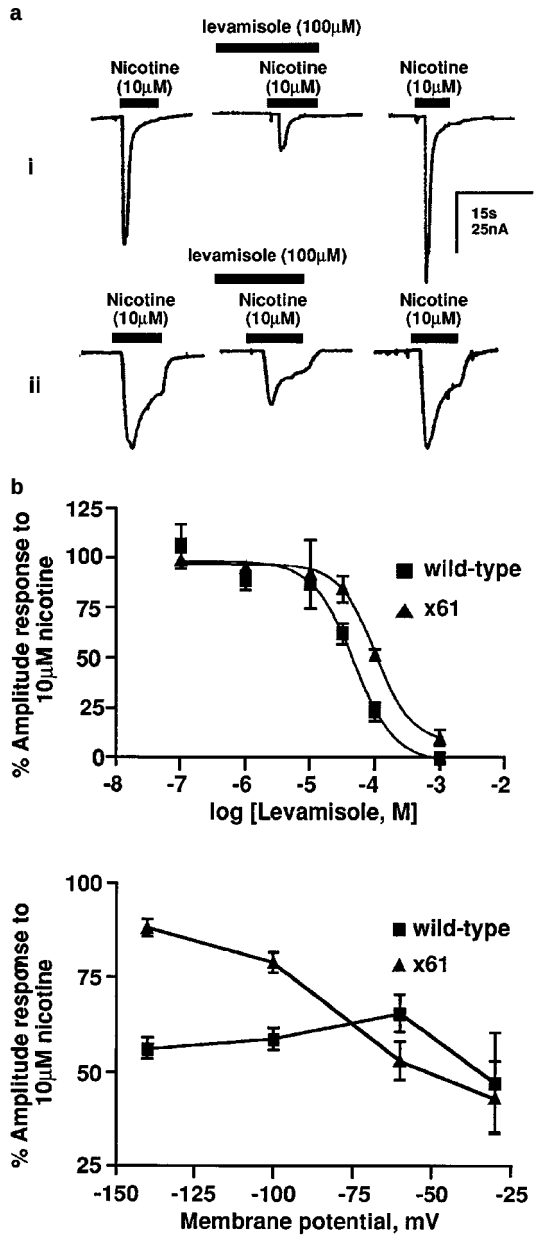

Figure 5 Levamisole block of nicotine-induced currents in wild-type and *x61* mutant α_7 nicotinic ACh receptors. (a) Levamisole (100 μM) reversibly reduced the amplitude of nicotinic responses in both the wild-type (trace i) and α_7*x61* mutant (trace ii) receptors. The experimental procedure was as for Figure 4a. (b) This action of levamisole was dose-dependent. The dose-dependence of the action of levamisole on the wild-type and α_7*x61* receptors was determined as follows: the amplitude of the response to 10 μM nicotine was measured, then after 2 min wash in normal saline the oocyte was bathed in saline containing levamisole at the test concentration, followed by a 15 s challenge with saline containing nicotine and antagonist. The amplitude of the latter response is expressed as percentage of the amplitude of the control response and plotted against the levamisole concentration. Each point represents the mean of at least three experiments, each on a separate oocyte, and vertical lines show s.e.mean. (c) The voltage-dependence of levamisole block of nicotine-induced responses in the wild-type and α_7*x61* receptors. The membrane was clamped at the test holding potential and the oocyte exposed to nicotine (10 μM). The oocytes were then washed for 2 min in normal saline then for 1 min in saline containing 3×10^{-5} M levamisole. The amplitude of the response to a challenge with saline containing nicotine and levamisole was then measured. Responses were normalized to the response to 10 μM nicotine and plotted against the clamped membrane potential. Each point represents the mean of at least three experiments, each on a separate oocyte, and vertical lines show s.e.mean.

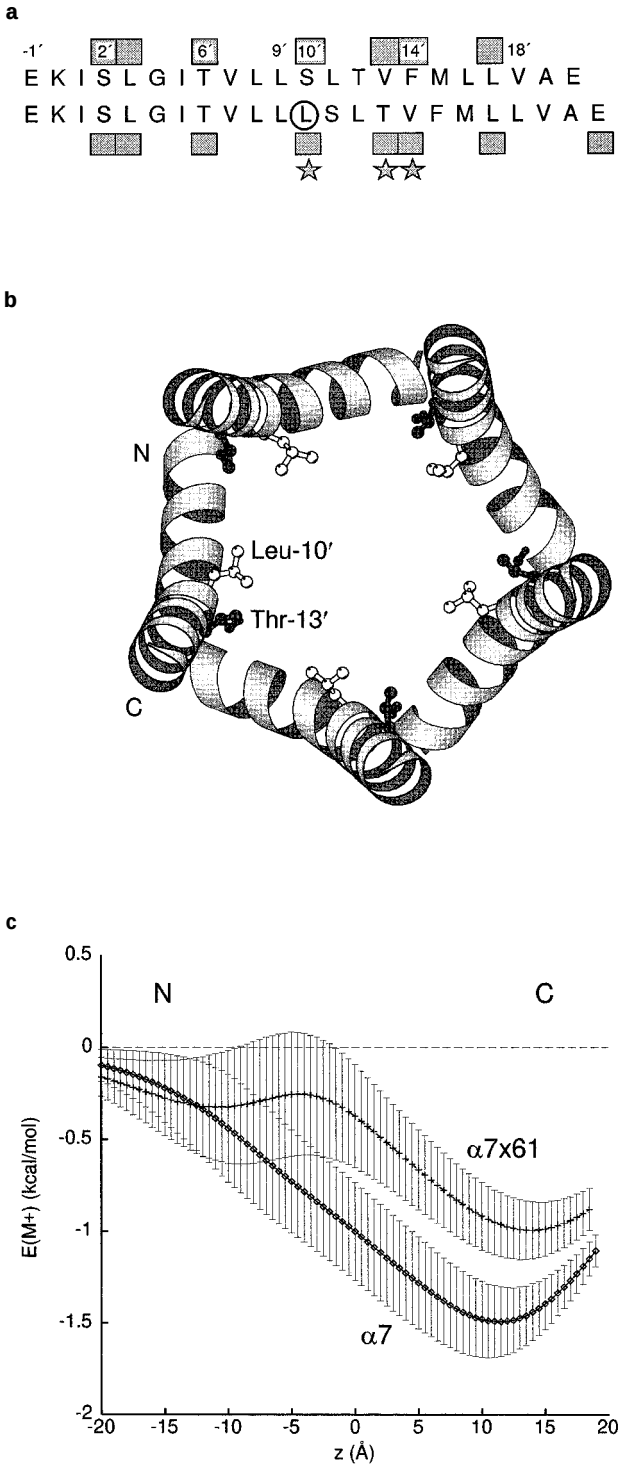

Figure 6 Changes in the predicted pore-lining residues resulting from the insertion of a leucine in the M2 helix of α_7. The structure of the pore-lining domain of α_7*x61* in the open conformation is compared to that of the wild-type (Sankararamakrishnan *et al.*, 1996). (a) Amino acid sequences of M2. Residues predicted to line the pore are marked by shaded boxes. The leucine insert is circled. Changes in the pore lining residues in the mutant are marked by stars. (b) A ribbon diagram of the model of the α_7*x61* channel viewed from the extracellular side. The changes of Ser10′ to Leu10′ and Val13′ to Thr13′ result in a non-polar side chain which projects into the pore at 10′ and a polar side chain at 13′. (c) The electrostatic potential energy profiles for an M^+ ion moved along the pore axis of the α_7 and α_7*x61* models. Negative values of z correspond to the intracellular mouth and positive values to the extracellular mouth, with z = 0 corresponding to the position of the sidechain of Leu9′. The vertical lines show s.d. either side of the potential energy derived by averaging across all 25 structures in each ensemble of models.

the non-polar side chain is reduced. In addition Glu20′ is moved so that it points more towards the lumen of the pore. However, the conformation of this side chain is quite variable in all of the α_7 channel models. These changes are illustrated in the ribbon diagram of the mutant pore shown in Figure 6b where side chains of the inserted leucine and Thr13′ can be clearly seen projecting into the pore. These changes in the structure also result in alterations to the predicted electrostatic potential energy profile along the pore axis (Figure 6c) such that the depth of the well in the C-terminal region is reduced and a 'hump' in the profile is introduced in the profile of the α_7*x61* channel. Such changes in the potential profiles may account for the small differences in current-voltage relationships observed between the mutant and wild-type (Figure 4b).

Discussion

We have studied the effects of inserting a leucine residue into M2, between Leu9′ and Ser10′, on the function of the chicken α_7 nicotinic ACh subunit. The insertion of an amino acid into M2 is an unusual alteration which does not, however, impair the ability of α_7 subunits to form functional receptors, but does significantly alter the properties of these receptors. We have modelled the effect of this change on the open channel. As the structure from Leu9′ towards the intracellular side of the channel is likely to be preserved due to the more extensive helix-helix constraints that exist that side of Leu9′ we assumed that any changes in the structure will occur, in the less constrained region, to the extracellular side of Leu9′. In this case we predict that a number of significant changes to the channel lining will occur, notably that position 10′ (Ser to Leu) will become non-polar whilst position 13′ (Val to Thr) will become polar.

The leucine insertion results in a channel with novel properties. Site-directed mutagenesis studies on α_7 receptors have identified a number of residues which influence desensitization, including residues in M2. These mutations in M2 (of Glu-1′, Thr6′, Leu9′ and Val13′) result in nicotinic ACh receptors that activate and desensitize slowly and exhibit an increased apparent affinity for ACh (Revah *et al*., 1991). Competitive antagonists such as DHβE and (+)-tubocurarine, which preferentially bind to and stabilize the desensitized state, act as agonists of these mutated α_7 channels (Bertrand *et al*., 1992b), a similar change in the effect of dimethyl-(+)-tubocurarine was observed in the human muscle α subunit Val7′Phe alteration. Such changes in behaviour have been explained in terms of alterations in the transition rate-constants between states (Galzi *et al*., 1992). Furthermore in α_7 receptors containing such mutations an additional conductance state is observed. These observations can be explained by the existence of a novel, desensitized but conducting state ((D*) as reviewed in Galzi *et al*., 1996). However, studies on muscle receptors with substitutions of Leu9′ have not identified an extra conductance state (Labarca *et al*., 1995; Filatov & White, 1995).

In the α_7*x61* protein both the rate of response onset and the rate of desensitization were reduced. However, in contrast to previous mutants, the agonist affinity is scarely affected and the competitive antagonist (DHβE) is not converted to an agonist. It is therefore unlikely that this effect can be explained adequately by the existence of the novel D* state in the α_7*x61* protein. A formal explanation is that the leucine insertion alters the stability of both the closed and open states, thus changing the onset and desensitization rates, but does not alter the relative stability of the two states and so does not change the apparent affinity for ACh or the behavior of competitive antagonists. Although there is no significant change in the value of pEC_{50} for nicotine we did observe a change in the shape of the curve. The most likely explanation for this is that it results from the substantial differences in the desensitization of the two receptors.

All single amino acid substitutions that alter desensitization, that have been examined thus far, also alter agonist affinity. The leucine insertion, even in our conservative model, produces changes at three positions in the channel lining alone. It is possible that in combination these alterations bring about approximately equal changes in the stability of both the open and closed states. Interestingly, Labarca *et al*. (1997) have shown that a Val13′Ser mutation in muscle nicotinic ACh receptors results in a significant increase in agonist affinity. In α_7*x61* Val13′ is replaced by a threonine. Despite the similarity of this change to the Val13′Ser substitution no effect on agonist affinity is observed. However, in the α_7*x61* model Ser10′ is replaced by the non-polar residue Leu, thus the overall polarity of this region of the structure is unaltered. The conservation of polarity within this region may underlie the lack of any alteration in the apparent agonist affinity of the receptor. Further speculation on the mechanism of this mutation awaits a better understanding of the effects of the leucine insertion on single channel properties and/or the structural basis of channel function.

The *lev-1 x61* allele in the nematode, *C. elegans*, results in worms which are extremely resistant to levamisole. The mutation in this allele results in the same mutation as that we have described above (Fleming *et al*., 1997) and indeed it was the discovery of this alteration which prompted our interests in the effect of insertions in M2. However, caution is needed in making even limited comparisons between α_7 and *C. elegans* nicotinic ACh receptor subunits such as LEV-1. LEV-1 is a non-α subunit, that is able to contribute to hetero-oligomeric nicotinic ACh receptors, when expressed in *Xenopus* oocytes, but is not functional as an homo-oligomer. That mutations in non-α subunits may still alter channel behaviour and also even effect apparent agonist affinity has been demonstrated by mutations involved in congenital myasthenic syndromes which occur in the β and ε subunits (e.g. Engel *et al*., 1996; Milone *et al*., 1997). Levamisole acts as an agonist on LEV-1-containing receptors in *Xenopus* oocytes (Fleming *et al*., 1997). The LEV-1 subunit is presumed to be part of the *C. elegans* muscle receptor (Lewis *et al*., 1980; 1987). In *Ascaris suum* both agonist and open-channel blocking actions of levamisole have been observed on muscle nicotinic ACh receptors (Robertson & Martin, 1993; Martin *et al*., 1996). Blocking actions are observed at higher levels of levamisole than those required for agonist action where this occurs. For example, 'flickering block' was observed in the *Ascaris suum* muscle preparations at 30–100 μM (Robertson & Martin, 1993), whilst Ballivet *et al*. (1996) determined an IC_{50} of 36 μM on the Ce21 subunit from *C. elegans* expressed in *Xenopus* oocytes. α_7 is a functional homo-oligomer on which the only action of levamisole is as a blocker. Nevertheless, it is of interest that the insertion of the leucine at this position results in a small reduction in the antagonist potency of levamisole on these receptors. In addition the action of levamisole becomes voltage-dependent in the mutant receptor. Martin and co-workers (1996) have proposed a two-site model for the blocking action of the related compound morantel on the nicotinic ACh receptors of *Ascaris suum* muscle. It is envisaged that both sites are located in the transmembrane region. Thus we can speculate that the

change in the voltage-dependence reflects changes in the channel, caused by our mutation, which alter one of these two binding sites or, perhaps, access to it. It will be interesting to ascertain whether in the native *C. elegans* receptor this mutation affects both agonist and antagonist actions of levamisole or only the latter. Understanding the effects of such mutations on the action of levamisole is important to understanding the antiparasitic action of this compound and mechanisms of resistance.

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