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Agonist activation and α-bungarotoxin inhibition of wild type and mutant α7 nicotinic acetylcholine receptors

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Abstract

The properties of wild type and mutant rat nicotinic α7 receptors expressed in *Xenopus* oocytes were investigated using electrophysiology and site-directed mutagenesis. When compared at individual agonist concentrations, neither the normalised nicotinic, nor acetylcholine, responses of the wild type receptors were significantly different from the corresponding responses obtained from a first extracellular domain mutant, phenylalanine^{189}tyrosine ($P > 0.05$). The dissociation constants (K_D) of the wild type (4.7 nM) and Phe^{189}Tyr mutant (5.2 nM) receptors for α-bungarotoxin were estimated by an electrophysiological approach. The similarity of the results suggests that the mutation did not lead to a widespread disruption of structure–function relationships, although a slight change in nicotine sensitivity may have occurred. In contrast, the mutations (Tyr^{190}Gln, first extracellular domain), (Glu^{261}Ala, M2 region) severely compromised receptor function. An additional mutation was made in a negatively charged motif of the second extracellular domain which is conserved in homomeric nicotinic receptors. This mutation, Asp^{268}Ala, also caused a loss of function. Thus the structure–function relationships in nicotinic α7 receptors have parallels with heteromeric nicotinic receptors, but there may also be some marked differences.

Keywords: Oocytes; Nicotinic; Receptor; α7; α-Bungarotoxin; Mutagenesis

1. Introduction

A wide diversity of neuronal nicotinic acetylcholine receptor subunits is involved in the production of excitatory ligand gated ion channels in the central nervous system (see Wonnacott, 1997, review). In total, nine α-subunits have been cloned and each shares the diagnostic features of vicinal cysteines at positions equivalent to positions 192, 193 in the *Torpedo* α-subunit. There are also three neuronal β-subunits, which share 40–70% homology with α-subunits, (Boulter et al., 1990; Séguéla et al., 1993). One member of this gene family, the neuronal nicotinic α7-subunit (Schoepfer et al., 1990), has attracted considerable scientific interest because of its ability to form functional homomeric receptors when expressed in *Xenopus* oocytes (Bertrand et al., 1992a; Séguéla et al., 1993). These studies also showed that nicotinic α7 receptors have a unique pharmacological profile, which includes an extreme sensitivity to the antagonist α-bungarotoxin, (α Bgt).

Functional nicotinic ligand gated ion channels with α7-type pharmacology have also been discovered in rat brain slices and in neurons freshly dissociated from rat brain tissue (Alkondon and Albuquerque, 1993; Freedman et al., 1993; Gray et al., 1996). However the nicotinic α7 subunit may participate in more than one form of native receptor, since both heteromeric and homomeric nicotinic α7-type receptors have been identified in rat brain tissue (Alkondon and Albuquerque, 1993; Albuquerque et al., 1995). Immunohistochemical localisation studies (Del Toro et al., 1994), α-bungarotoxin binding studies (Clarke et al., 1985) and in situ hybridisation studies (Séguéla et al., 1993) suggest that nicotinic α7-type receptors are present in the hippocampus, hypothalamus, cerebral cortex, inferior colliculus, interpeduncular nucleus, pons, medulla and regions of the brain stem. Within neurons, nicotinic α7

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subunits can be found in cell soma, somatodendritically, presynaptically and preterminally (Arcava et al., 1987, Albuquerque et al., 1997, Wonnacott, 1997, review).

The pharmacological properties of the nicotinic α7 receptor have become the focus of great clinical interest. This is because mutations of the nicotinic α7 gene are associated with schizophrenia (Freedman et al., 1997) and epilepsy (Emslie et al., 1997). There is also evidence that nicotinic α7-type receptors may be specifically involved in mediating nicotine's effects in smoking-related diseases. Examples include heart disease (Townsend and Meade, 1979), osteoporosis (McKinlay et al., 1985; Benowitz, 1996) and lung cancer (Schuller, 1994; Schuller et al., 1990; Codignola et al., 1994). Therefore the nicotinic α7-type receptor is a possible target for several types of therapeutic drug. Knowledge of the way in which specific ligands interact with particular amino acids on nicotinic α7 subunits may therefore assist in the design of drugs used to intervene in the above conditions.

We have therefore used site-directed mutagenesis and electrophysiological methods to investigate the possible influence of various amino acids on the pharmacological properties of rat nicotinic α7 receptors. The identity of these amino acids may be different from those involved in ligand binding for heteromeric receptors containing muscle type α1 or neuronal α2–α4 receptors. For heteromeric nicotinic receptors such as the mouse muscle α1-containing receptors, negatively charged amino acids in the first extracellular domain of non-α subunits (e.g., δ) can contribute to the pharmacological properties of the receptors (Czajkowski et al., 1993). Among the neuronal heteromeric receptors such as α3β4, the substitution of the β4 subunit for the β2 subunit can greatly affect ligand–receptor interactions (Duvoisin et al., 1989, Cachelin and Rust, 1994). Since there are no non-α subunits in a homomeric nicotinic α7 receptor, the amino acid determinants of ligand sensitivity must be entirely provided by a single subunit, and may therefore be different to those occurring in heteromeric receptors. The target sites for the site-directed mutagenesis pursued here were therefore chosen to investigate whether or not the structure–function relationships of α7 subunits in homomeric nicotinic receptors are essentially similar to those of other α subunits in heteromeric nicotinic receptors. Residues in the first extracellular domain, M2 region and second extracellular domain of the nicotinic α7 subunit were investigated.

In the first extracellular domain, the nicotinic α7 subunits of mouse, rat, human and chicken all have a phenylalanine residue at position 189, and a tyrosine residue at position 190. All these nicotinic α7 receptors tend to be substantially more sensitive to nicotine than to acetylcholine. The nicotinic α1 subunits of muscle type receptors also have a phenylalanine residue at position 189, but are less sensitive to nicotine than to acetylcholine. For example, the potency ratio of acetylcholine to nicotine at the mouse muscle α1β1γδ receptor expressed in oocytes is 100:1, (Connolly, unpublished results). Like α7, chick nicotinic α8 subunits can also form homomeric receptors but have a tyrosine residue at position 189. Nicotinic α8 receptors are about equally sensitive to acetylcholine and nicotine, but compared to nicotinic α7 receptors have lower EC_{50}'s (about 1–3 μM for both agonists), (Gerzanich et al., 1994; Gotti et al., 1994). Unusually for an α-bungarotoxin binding receptor, the α9 homomeric receptor has a polar serine residue at position 189. This nicotinic receptor subtype does not respond to nicotine at all (Elgoyhen et al., 1994), but does have an EC_{50} of 10 μM for acetylcholine. Thus, among homomeric receptors, alterations of the tyrosine at position 189 or its equivalent are correlated with changes in nicotine sensitivity. To investigate the influence of this residue in the nicotinic α7 receptor, the phenylalanine at position 189 was mutated to tyrosine. In heteromeric receptors, the tyrosine at position 190 of nicotinic α1 subunits has previously been shown to have a major influence over pharmacological properties (e.g., Chen et al., 1995). To confirm that this is also the case with nicotinic α7 receptors, the tyrosine at position 190 was mutated to glutamine.

Amino acids in the M2 domain of subunits in heteromeric receptors are thought to form rings of negative charge which line the walls of the central ion pore of nicotinic receptors, (Konno et al., 1991). In this study, glutamate^{261} was mutated to alanine to confirm that the integrity of this feature was also important to function in nicotinic α7 receptors.

Finally a novel feature of homomeric nicotinic receptors was investigated. In the second extracellular domain, nicotinic α7 subunits contain the strongly anionic motif serine–aspartate–serine (SDS). It seems possible that this motif fulfils the same function for homomeric receptors as anionic residues of non-α subunits do for heteromeric receptors. To test whether this motif might have some functional importance, the central aspartate residue, aspartate^{268}, was mutated to alanine.

2. Materials and methods

2.1. Mutagenesis

The rat nicotinic α7 cDNA was kindly supplied by Professors Jim Boulter (UCLA, USA) and Steve Hienemann, (Salk Institute, USA). Site-directed mutagenesis was carried out using the protocol provided by the Muta-Gene Phagemid In Vitro Mutagenesis Kit Instruction Manual, Bio-Rad. Four mutations were made: phenylalanine^{189} was mutated to tyrosine, (Phe^{189}Tyr); tyrosine^{190} to glutamine, (Tyr^{190}Gln); glutamate^{261} to alanine (Glu^{261}Ala) and aspartate^{268} to alanine (Asp^{268}Ala). The amino acids are numbered relative to the two adjacent cysteines at positions 192, 193. The nucleotide sequences of the wild type and mutated clones were verified by sequencing.

2.2. Functional expression in Xenopus oocytes

Defolliculated oocytes were prepared for injection as described previously (Covernton and Connolly, 1997). Wild type rat nicotinic α7 cRNAs were transcribed from the corresponding cDNA subcloned into either the pcDNA1/Amp or the pGEMHE vector (Liman et al., 1992). Mutant rat cRNAs were all transcribed from the corresponding clones in the pcDNA1/Amp vector. The wild type and mutant rat nicotinic α7 cRNAs (20–40 ng per oocyte, cytoplasmic injection) or constitutively expressing cDNA plasmid (20–200 ng per oocyte, nuclear injection) were introduced into the oocytes. The oocytes were then incubated for 2–4 days at 19.5°C before electrophysiological experiments took place.

2.3. Electrophysiological recording

Electrophysiological experiments were carried out using an Axon Geneclamp 500 to obtain two-electrode voltage clamp recordings ($V_H = -60$ mV, unless otherwise stated) of the agonist responses of injected oocytes. Current and voltage electrodes were manufactured from borosilicate glass (GC150-TF, Clark Electromedical) with 0.5–1 MΩ and 2–4 MΩ resistances respectively. Current electrodes were filled with CsCl (0.25 M), CsF (0.25 M) and EGTA (100 mM) pH 7.2, while voltage electrodes were filled with KCl (3 M). Oocytes were perfused at 20–30ml/min with an external recording solution of the following composition: NaCl 115 mM, $BaCl_2$ 1.8 mM, KCl 2.5 mM and HEPES 10 mM with the pH adjusted to 7.2. The $BaCl_2$ was substituted for the more usual $CaCl_2$ in the recording solution in order to minimise the activation of Ca^{2+}-activated chloride conductances in the oocytes. Atropine (1 μM) was present throughout these experiments in order to block endogenous muscarinic responses. Drugs were applied for 5–8 s and responses were recorded onto a chart recorder (Gould Easygraph). For the experiments with α-bungarotoxin (provided by Prof. Jim Patrick, Baylor, USA), bovine serum albumin (0.1 mg/ml) was used as a carrier to prevent nonspecific toxin binding. In these experiments, the bovine serum albumin was also present in the control solution containing agonist in case the bovine serum albumin itself should have some effect on response height, (although this was not observed in preliminary experiments). Nicotine hydrogen tartrate, acetylcholine and other chemical reagents were supplied by Sigma-Aldrich, (UK). The nicotine hydrogen tartrate was buffered at high concentrations to avoid causing a change in the pH of the recording solution. In both control and test recordings of nicotinic currents, it was found to be critical to keep the rate of perfusion within the bath constant and as fast as possible (perfusion of the bath was complete within 1.5 s). This is because the rapid desensitisation of nicotinic α7 receptors means that with slow perfusion rates, there will be some desensitisation before the peak of the current response is attained. If the perfusion rate varies, so will the peak response. This led to the loss of some data in preliminary experiments.

2.4. Recovery from desensitisation

The effects of divalent metal ions on the time course of recovery of nicotinic α7 receptors from desensitisation were examined. To do this, responses to 30 μM nicotine were obtained with either 1.8 mM $CaCl_2$ or 1.8 mM $BaCl_2$ in the external recording solution. For each oocyte, test responses obtained at a variety of time intervals after a control response were expressed as a fraction of that control response. The results obtained from 4 oocytes in the solution containing 1.8 mM were then pooled together. A separate pool of data from 4 different oocytes in Ba^{2+} solution was also collated. These fractional test responses were plotted against time and the data fitted with a single exponential component according to the following equation:

$$I_t = I_{max}(1 - e^{-t/\tau})$$

Here I_t = fraction of the maximum (control) current at time t, t = time of observation, I_{max} = the maximal current (i.e., control = 1) and τ = time constant (min) of the recovery from desensitisation.

2.5. Concentration–response curves

Nicotine and acetylcholine responses were normalised to standard concentrations of 10 μM nicotine and 100 μM acetylcholine respectively. These standard concentrations of agonist were applied at various times during the experiment to check that the responses were not running down or desensitised. The averaged normalised responses (±S.E.M.) were plotted against the log of the concentration of agonist. The concentration–response curves were then fitted by the method of least squares, (weights equal) to the Hill equation:

$$I = I_{max}\left[\frac{(X)^{n_H}}{(X)^{n_H} + (XC_{50})^{n_H}}\right]$$

where I = observed current response, I_{max} = maximal current response, X = agonist concentration, n_H = Hill coefficient and XC_{50} = the concentration of agonist which elicits a 1/2 maximal response.

2.6. Estimation of the K_D for α-bungarotoxin binding

The theoretical basis of this electrophysiological method for determining the dissociation constant, K_D of a high affinity antagonist has been described in detail by Bowman and Rand (1980) and Jenkinson (1996). A similar approach has also been applied to the study of the binding of scorpion toxin to potassium channels (Gross et al., 1994),

the binding of ω-conotoxin-GVIA to N-type Ca^{2+} channels (Ellinor et al., 1994) and methyllycaconitine to chick nicotinic α7 receptors (Palma et al., 1996).

The experimental protocol involves the measurement of decreasing responses of nicotinic α7 receptors to nicotine as they are progressively blocked by α-bungarotoxin. Initially, a concentration of 30 μM nicotine was applied to the oocyte at 5-min intervals until three reproducible responses were obtained. This protocol did not lead to run down of the receptor responses and was therefore used in all the experiments. Five minutes after the final control response had been washed off, the perfusing solution was switched to one which contained either 3 nM, 1 nM or 300 pM α-bungarotoxin. Two minutes after the perfusion of a particular concentration of toxin had begun, (a total of 7 min after the previous control agonist response), the preparation was again challenged with an external solution containing 30 μM nicotine and the appropriate concentration of α-bungarotoxin. It was assumed that an agonist response at the time that the toxin was added ($t = 0$ min) would be the same as the average of the three previous control responses. Again it was found important to keep the flow rate of all the test solutions equal and constant. Following this initial agonist + toxin application, the test solution was then washed out in an external solution containing only α-bungarotoxin. The toxin was therefore continuously present in the bathing solution and progressively bound to a larger and larger fraction of the receptors (see Fig. 7). Nicotine (30 μM) was then repeatedly co-applied with α-bungarotoxin every 5 min until a period of 25 min had elapsed. Since the binding of α-bungarotoxin was very slow to reverse (> 1 h), only the rate of onset of inhibition was measured and only one concentration of α-bungarotoxin could be studied in any given experiment.

At each time point, the remaining agonist-induced current was expressed as a fraction of the mean of the initial control agonist responses for each cell obtained in the absence of toxin. This fractional inward current was plotted against time and the data fitted to the following equation using software supplied by David Colquhoun (University College London, http://www.ucl.ac.uk/Pharmacology/dc.html):

$$Y = A + (1 - A)e^{-t/\tau}$$

where Y is the fractional current at time t following the application of antagonist, A is the residual current after a very long period when the binding has reached equilibrium, and τ is the time constant for the development of current inhibition. The time constant obtained in this way was then converted to a rate constant for the onset of current inhibition, k_{on}, which is sometimes called the 'on-rate'. k_{on} is equivalent to $1/\tau$, i.e., 1/time constant of the current inhibition. All the k_{on} values at a particular toxin concentration were then averaged. In this way, a mean (±S.E.M.) 'on-rate' was derived for each concentration of α-bungarotoxin used at the wild type and mutant 1214 receptors. These mean 'on-rate' values were then plotted on linear axes against their respective concentrations of α-bungarotoxin. The data was fitted to a straight line using the following equation by the least squares method:

$$k_{on} = \frac{1}{\tau} = k_{+1}[\alpha\,\mathrm{Bgt}] + k_{-1}$$

where k_{+1} is the association rate constant for the binding of α-bungarotoxin to nicotinic α7 receptors, and k_{-1} is the dissociation rate (see Fig. 8). The value of k_{+1} was obtained from the slope of the graph, whereas the value of k_{-1} could be calculated as the intercept of the fitted straight line on the y axis. The equilibrium dissociation constant, K_A, for the binding of α-bungarotoxin to the nicotinic α7 receptor can then be calculated from $K_A = k_{-1}/k_{+1}$.

Student's t-test was used to determine the statistical significance of the results (with the more stringent Welch correction where appropriate). n-Values refer to the number of oocytes in which the experiments were performed unless otherwise stated. Means are given ±S.E.M. unless otherwise stated.

3. Results

3.1. Time course of resensitisation

The time course for the resensitisation of wild type nicotinic α7 homomeric receptors after exposure to nicotine (30 μM) was examined with either calcium or barium as the divalent cation in the external recording solution. Oocytes were initially tested with at least three control applications of 30 μM nicotine at 5-min intervals until a reproducible control response had been established. Next a pair of responses to nicotine (control and test) were obtained with time intervals ranging from 1 to 5 min between the applications. In between each pair of applications (control + test), the preparation was allowed to recover for 5 min whilst continually being washed with external solution. This allowed the responses to recover to their control levels and checked that they were not running down during the time course of the experiment. Fig. 1 shows that as the time interval between the control and test applications is increased, the degree of recovery increases. For each oocyte, the test responses were expressed as a fraction of the original control responses. It proved difficult to obtain the data points in different oocytes at exactly the same time, so for each ionic solution, the results from four oocytes were pooled together. Fig. 2a. shows a scatter plot of fractional currents against the time interval between applications obtained from four oocytes in 1.8 mM Ca^{2+} recording solution. The time constant for the recovery of the response was found to be 2.4 min. This was similar to the time constant of 2.2 min obtained for the pooled data for the recovery of responses in four oocytes which were

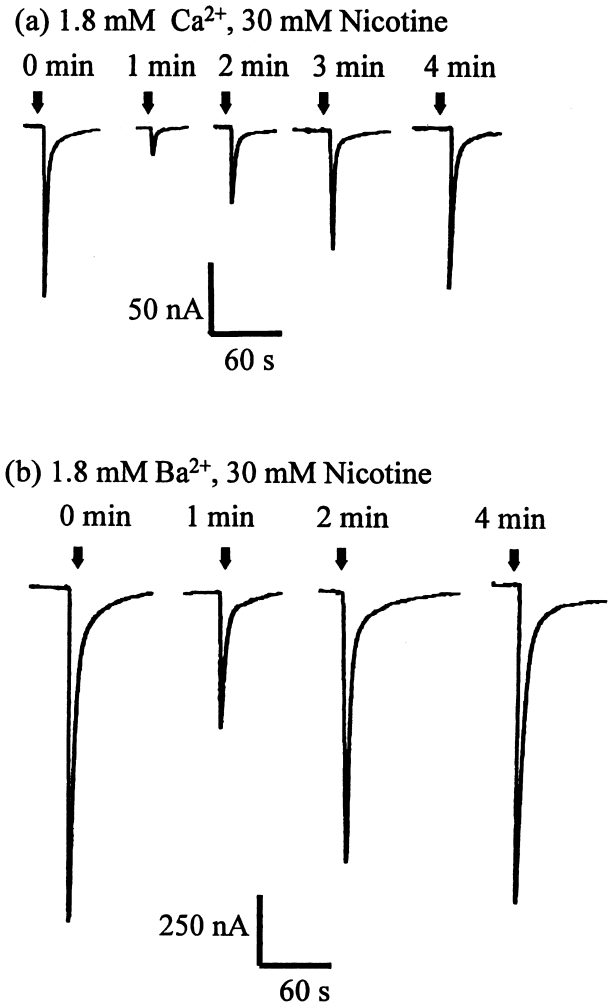

Fig. 1. The recovery of inward current responses elicited by 5–7 s applications of 30 μM nicotine as the interval between applications is increased. The response at 0 min is representative of a control response. The elapsed time between control and test responses is indicated above each trace. Oocytes expressing wild type nicotinic α7 homomeric receptors were perfused with (a) extracellular solution containing 1.8 mM Ca^{2+} (b) extracellular solution containing 1.8 mM Ba^{2+} in place of Calcium.

perfused with 1.8 mM Ba^{2+}-containing solution (Fig. 2b). Overall, it appears that the wild type nicotinic α7 homomeric receptor has a similar rate of resensitisation in both calcium and barium containing solutions. These experiments also showed that following an initial application of 30 μM nicotine, the response height was restored to its initial value within 5 min. Therefore a wash time of 5 min was adopted between agonist applications of 30 μM nicotine in subsequent toxin binding experiments, (see below).

3.2. Responses of wild type and mutant ($Phe^{189}Tyr$) α7 receptors to nicotine

As expected of wild type nicotinic α7 receptors, the inward current responses to applied nicotine were rapidly activated and inactivated (Fig. 3a). The application of increasing concentrations of nicotine from 1 μM to 1 mM resulted in inward current responses whose magnitude increased with increasing concentration. In 14 oocytes, the current response to a standard concentration of nicotine (10 μM) was obtained. The magnitudes of these inward current responses to 10 μM nicotine ranged from 9 to 144 nA (mean ± S.E.M., 123.3 ± 45.0 nA, $n = 14$). For each oocyte, responses to other concentrations of nicotine were expressed as a percentage of these standard responses and the results pooled. The resultant concentration–response

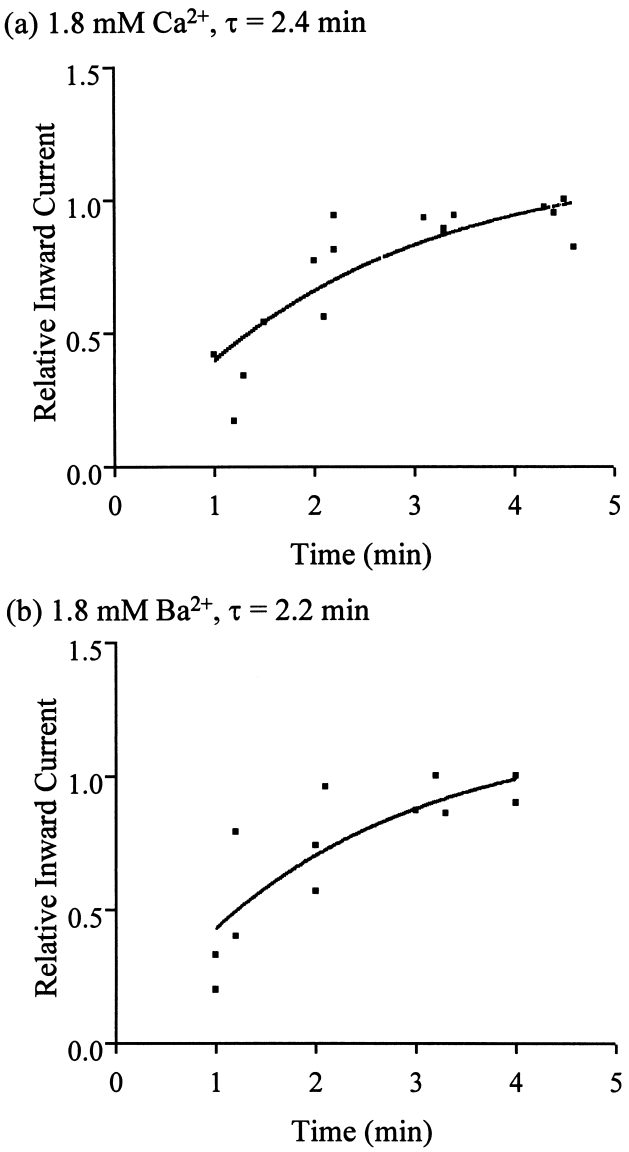

Fig. 2. The time-dependent recovery of currents evoked by 30 μM nicotine at various time points after an initial control response. Individual data points represent fractional inward current responses compared to control. Data has been pooled from four individual oocytes. Current responses are plotted as a function of time for (a) extracellular solution containing 1.8 mM Ca^{2+} (b) extracellular solution containing 1.8 mM Ba^{2+}.

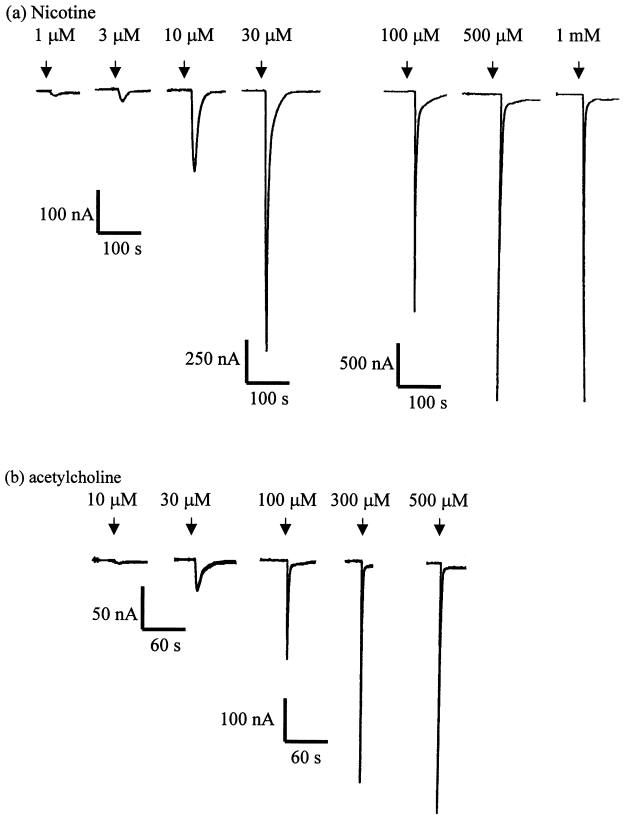

Fig. 3. Inward current responses elicited by various concentrations of agonist acting at wild type nicotinic α7 receptors. The upper panel (a) illustrates the responses to nicotine, while the lower panel (b) illustrates the responses to acetylcholine from a different oocyte. Black arrows indicate the application of agonist for 5 s with intervals of 5 min between applications. Note that the scales are reduced for the higher agonist concentrations.

relationship was fitted with the Hill equation and is shown in Fig. 5 (open circles). A Hill coefficient of 1.0 and an EC_{50} value of 50 ± 20.9 μM (±S.E.M. of estimate) were obtained.

The above responses obtained from the nicotinic α7 wild type receptor were compared with those obtained from a mutant nicotinic α7 receptor (Fig. 4). In the subunits of the mutant receptor, the nonpolar phenylalanine residue at amino acid 189 (relative to the paired cysteines at 192, 193) had been replaced by negatively polar tyrosine residue ($Phe^{189}Tyr$). The responses of the $Phe^{189}Tyr$ mutant receptor to μM concentrations of nicotine (Fig. 4a) were similar in time-course to the responses produced by the wild type nicotinic α7 homomeric receptor. It was also found for the mutant receptor that a 5-min wash was sufficient to allow complete recovery of the response to 10 μM nicotine (data not shown). Thus the resensitisation properties of the $Phe^{189}Tyr$ mutant receptor appear similar to those of wild type nicotinic α7 receptors, but given the practical problems of measuring changes in very fast rates of desensitisation and recovery in oocytes, such a conclusion must be treated with caution. As with the wild type nicotinic α7 receptors, 10 μM nicotine was used as a normalising standard for the $Phe^{189}Tyr$ mutant to obtain the concentration–response plot shown in Fig. 5 (closed circles). For the $Phe^{189}Tyr$ mutant, the EC_{50} value was (74.1 ± 39 μM) and the Hill coefficient was 0.8. These results do not suggest that there is a significant difference between the concentration–response characteristics of wild type and mutant receptors and when compared at individual concentrations, a significant difference is not found ($P > 0.05$). Nonetheless, the concentration–response relationship of the mutant response does not superimpose upon the wild type response (Fig. 5). Also, while the normalised responses to 300 and 500 μM nicotine were significantly greater than those to 30 μM nicotine for the wild type receptor, ($P < 0.002$), this was not the case for the $Phe^{189}Tyr$ mutant ($P > 0.05$). Thus it is still possible that there is a slight reduction in nicotine sensitivity for the mutant.

3.3. Responses of wild type and mutant ($Phe^{189}Tyr$) nicotinic α7 receptors to acetylcholine

Applications of acetylcholine to a separate set of oocytes expressing the wild type nicotinic α7 homomeric receptor also led to rapidly activated and inactivated responses, (Fig. 3b). In these experiments, 100 μM acetylcholine was

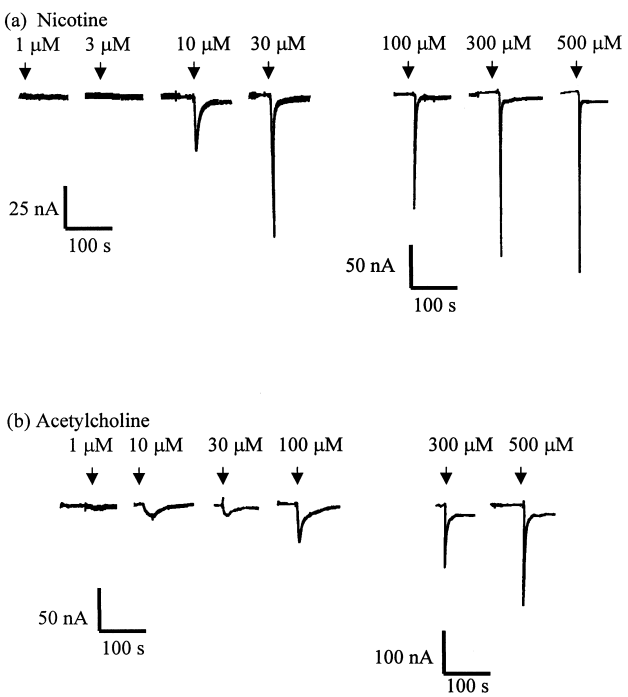

Fig. 4. The increase in inward current responses elicited by various concentrations of agonist acting at mutant $Phe^{189}Tyr$ nicotinic α7 receptors. The upper panel (a) illustrates the responses to nicotine while the lower panel (b) illustrates the responses to acetylcholine from a different oocyte. Black arrows indicate the application of agonist for 5 s with intervals of 5 min between applications. Note that the scales are reduced for the higher agonist concentrations.

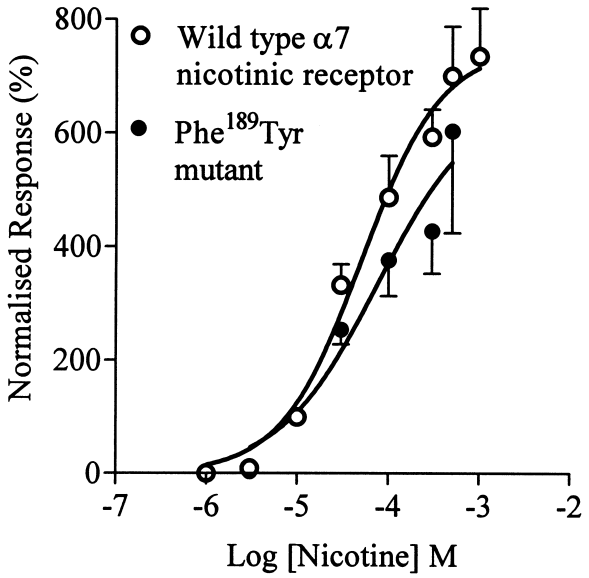

Fig. 5. Concentration–response relationships for the response to nicotine of wild type nicotinic α7 receptors (open circles, ○, $n = 7–14$ oocytes at each concentration) and Phe[189]Tyr mutant nicotinic α7 receptors (closed circles, ●, $n = 5–6$ oocytes at each concentration). In this case the of the fitted lines are not superimposable.

used as a normalising concentration because concentrations below this induced only very small inward currents. The range of current responses obtained from 100 μM acetylcholine was between 8 nA to 154 nA (mean ± S.E.M. = 107.7 ± 22.5 nA, $n = 15$). The acetylcholine concentration–response relationship for the wild type receptor is shown in Fig. 6 (open circles). The Hill coefficient for acetylcholine was 1.3 and the EC_{50} was 254 ± 31.9 μM.

Fig. 4b shows responses to acetylcholine of a different set of oocytes which were expressing the Phe[189]Tyr mutant receptor. As with the wild type receptor, responses were normalised to those obtained with 100 μM acetylcholine. The concentration–response curve for the responses of the Phe[189]Tyr mutant to applied acetylcholine are shown in Fig. 6 (closed circles). An EC_{50} value of 217 ± 42 μM, (± S.E.M.) and a Hill coefficient of 1.1 were obtained. These results appear very similar to the wild type concentration–response characteristics, and the concentration–response curves are superimposable, suggesting that the sensitivity of the receptor to acetylcholine has not been much altered by the mutation. However due to the variability of the acetylcholine response at high concentrations of acetylcholine, the EC_{50} value is not well defined, and so a small change may have taken place.

3.4. Comparison of the K_D for the binding of α-bungarotoxin to wild type and mutant receptors

We also decided to test whether the Phe[189]Tyr mutation might affect the interaction of the nicotinic α7 receptors with antagonists. A competitive, large peptide, antagonist is expected to have several, separated points of interaction with the receptor. It will therefore probe a different set of receptor–ligand interactions to those of small agonist molecules. If the mutation brings about a widespread conformational change in the agonist binding domain of the receptor, then it might be expected to affect the binding of a competitive peptide antagonist. If instead, the effects of the mutation are quite localised, then the binding of the peptide may not be affected as long as it does not directly interact with the site of the mutation.

The competitive peptide antagonist chosen for this study was the nicotinic α7 receptor selective antagonist α-bungarotoxin. It has the advantage that its K_D can be measured electrophysiologically by the approach described in the Materials and methods section. Fig. 7a shows the first part of the experiment for the wild type nicotinic α7 receptor. There is a progressive inhibition of the response to a standard concentration of nicotine (30 μM) applied at 5-min intervals after continuous perfusion of the oocytes with 3 nM α-bungarotoxin. The inhibited responses are expressed as a fraction of the original response to 30 μM nicotine plotted against time of incubation with α-bungarotoxin and fitted with a single exponential component. From these inhibition plots, values for the rate of onset of the inhibition (on-rate or k_{on}) were obtained as described in the Materials and methods section. The rates of inhibition of the inward current responses evoked by 30 μM nicotine were measured and averaged for each of three different concentrations of α-bungarotoxin. At the lowest concentration of toxin (300 pM), acting at the wild

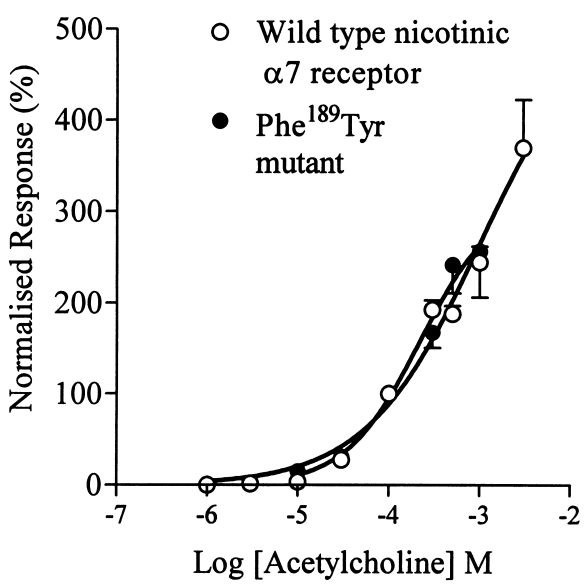

Fig. 6. Concentration–response relationships for the response to acetylcholine of wild type nicotinic α7 receptors (open circles, ○, $n = 8–14$ oocytes at each concentration) and Phe[189]Tyr mutant nicotinic α7 receptors (closed circles, ●, $n = 4–8$ oocytes at each concentration). The fitted lines appear to be approximately superimposable.

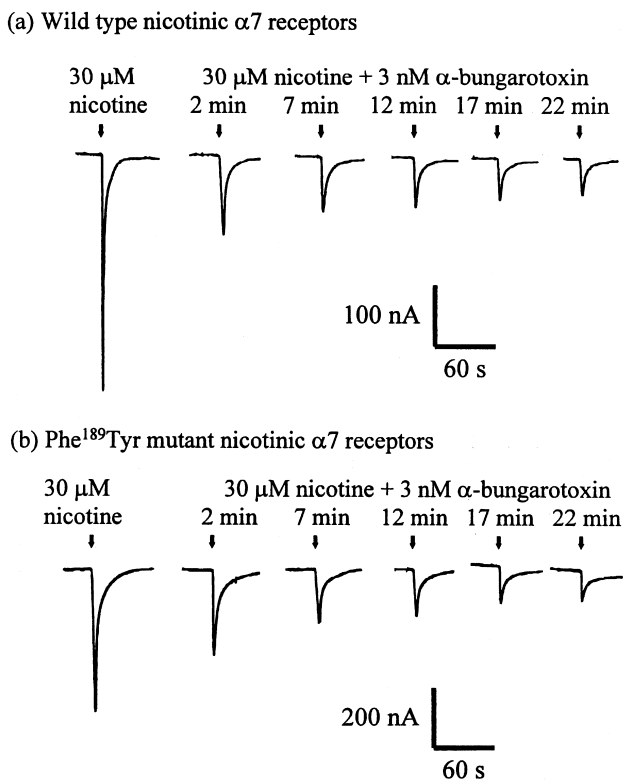

Fig. 7. The inhibitory effect of α-bungarotoxin (3 nM) on inward current responses to applied nicotine (30 μM). Control responses were applied at 5-min intervals until a standard response was obtained. Then, at a time point 5 min after the last control drug application, extracellular solution containing α-bungarotoxin was perfused through the chamber. Two minutes after that perfusion had begun, toxin and nicotine were repeatedly co-applied to the oocyte at 5-min intervals. The black arrows indicate the co-application of agonist with antagonist for 5 s. α-Bungarotoxin was continuously present in the wash solution between agonist applications. The upper panel (a) illustrates the inhibitory effect of α-bungarotoxin at the nicotinic α7 homomeric receptor, while the lower panel (b) illustrates the inhibition of the nicotinic α7 Phe[189]Tyr mutant receptor.

type receptor, the mean k_{on} rate was 0.2 ± 0.05 min^{-1} ($\pm$S.E.M., $n = 6$).

For 1 nM and 3nM α-bungarotoxin, the mean k_{on} values were 0.21 ± 0.035 ($n = 5$) min^{-1} and 0.30 ± 0.074 min^{-1} ($n = 6$) respectively. Thus the rate of onset of inhibition appeared to be proportional to the concentration of α-bungarotoxin.

To obtain the equilibrium dissociation constant K_D for the binding of the toxin to the receptor, the mean k_{on} values (equivalent to $1/\tau$) were plotted against the concentration of α-bungarotoxin (Fig. 8a).The data was fitted to a straight line and k_{+1} (the microscopic association rate constant for the binding of the toxin to the receptor) was measured as the slope of the plot, while k_{-1} (the microscopic rate constant for the dissociation of the antagonist from the receptor) was obtained from the calculated intercept on the y axis. The equilibrium dissociation rate constant, K_D, is then equal to the ratio k_{-1}/k_{+1}. The K_D value obtained from this plot was 4.7 nM.

The rate of onset of inhibition of inward current responses elicited by nicotine (30 μM) in the presence of three concentrations of α-bungarotoxin was also measured for the mutant Phe[189]Tyr receptor. Inhibition of the inward current responses elicited by Phe[189]Tyr after the application of 3 nM α-bungarotoxin is shown in Fig. 7b. Again the fractional responses to 30 μM nicotine were plotted against the duration of perfusion with α-bungarotoxin and the data fitted with a single exponential curve. At the lowest concentration of α-bungarotoxin, (300 pM), the mean on-rate for inhibition (k_{on}) was 0.29 ± 0.055 min^{-1} ($n = 5$). At 1 nM and 3 nM α-bungarotoxin, the k_{on} values were 0.36 ± 0.044 ($n = 4$) and 0.45 ± 0.088 ($n = 4$) min^{-1} respectively. As for the wild type receptor, the

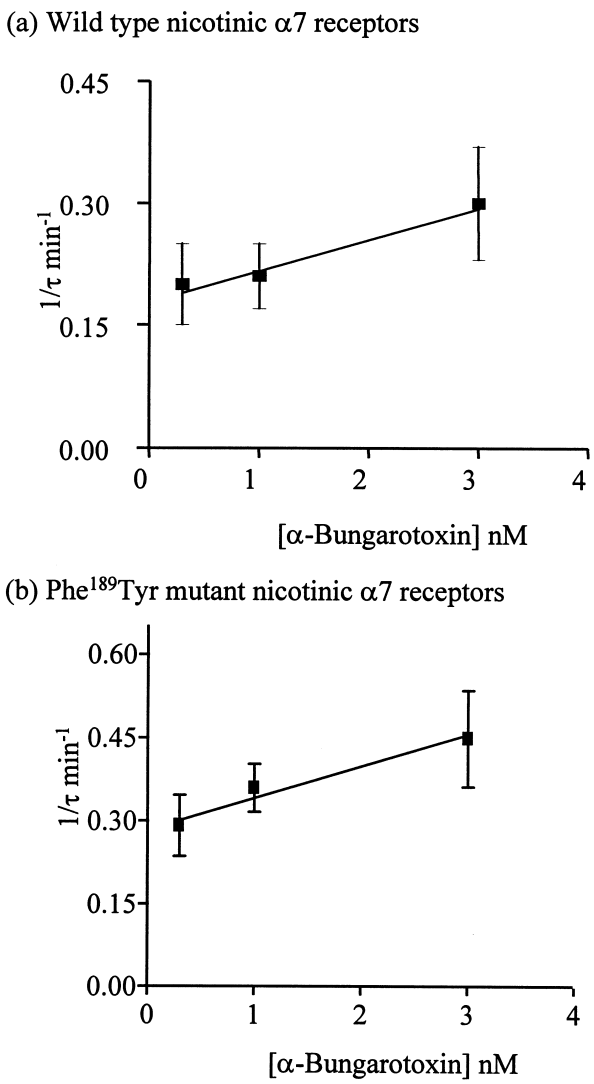

Fig. 8. Plot of mean reciprocal time constants of the inhibition of the 30 μM nicotine response against the concentration of α-bungarotoxin. Each data point represents the mean of four to six responses elicited by receptors in individual oocytes. Straight lines were fitted by linear regression analysis. The upper panel (a) shows the plot for inhibition of the wild type nicotinic α7 receptor, while the lower plot (b) shows the inhibition of the Phe[189]Tyr mutant nicotinic α7 receptor.

magnitude of k_{on} appeared to be directly proportional to the concentration of α-bungarotoxin for the Phe[189]Tyr mutation. Fig. 8b shows the plot of mean k_{on} against α-bungarotoxin for the mutant Phe[189]Tyr. A straight line was fitted to the plotted points and the K_D calculated as before. A value of 5.2 nM was obtained.

The overall K_D value for the mutant receptor is thus very similar to that of the wild type receptor. Furthermore, although the slopes and calculated *x*-intercepts of the lines in Fig. 8a and b are slightly different, comparison of the results for wild type nicotinic α7 receptors and mutant nicotinic α7 receptors using a two-sample Student's *t*-test revealed that there was no significant difference ($P > 0.05$) between the on-rates of inhibition for each receptor type at any of the three concentrations of toxin used: 300 pM ($P > 0.05$), 1 nM ($P > 0.05$) and 3 nM ($P > 0.05$). This suggests that the affinity of the mutant Phe[189]Tyr receptor is not significantly different to that for the wild type nicotinic α7 receptor to α-bungarotoxin. The result also implies that the Phe[189]Tyr mutation has not resulted in major disruption of the nicotinic α7 receptor structure.

3.5. Additional nicotinic α7 subunit mutations

Preliminary results were obtained for three other nicotinic α7 receptor homomeric mutants: tyrosine[190] was mutated to glutamine (Tyr[190]Gln), glutamate[261] was mutated to alanine (Glu[261]Ala) and aspartate[268] was mutated to alanine (Asp[268]Ala). These particular experiments were carried out at −100 mV in order to increase the chances of detecting functional responses.

The mutant Glu[261]Ala subunit did not produce a functional receptor when expressed singly in more than 30 *Xenopus* oocytes. Four separate batches of oocytes were used, and several different preparations of RNA. The RNA appeared of good quality when visualised on formaldehyde gel, and parallel preparations and expression of wild type nicotinic α7 subunit RNAs were successful. Despite these efforts, and the injection of large amounts of RNA and DNA, no functional expression of the mutant was detected. This suggests that the mutation itself has compromised the function of the receptor.

However, the other two mutant receptors, Tyr[190]Gln and Asp[268]Ala, were functional when expressed in *Xenopus* oocytes, although it required almost millimolar concentrations of acetylcholine and nicotine to activate them, (data not shown). Again this was despite the RNA appearing to be of good quality. In four cells in which expression was obtained, the application of 2 mM nicotine to the Tyr[190]Gln mutant produced inward current responses in the range 10–200 nA. The threshold concentration was 500 μM for nicotine, but it required millimolar concentrations of acetylcholine in order to obtain a response. A similar threshold for the response to nicotine was found for the Asp[268]Ala mutant receptor. In five cells where a response was obtained, the response to 1 mM nicotine ranged from 5 to 25 nA. Despite several attempts with different batches of RNA and oocytes, the quality of the functional expression did not change. It therefore seems these latter mutations severely compromise the function of the nicotinic α7 receptor. Unfortunately, because of the poor quality of the functional expression, it was not possible to analyse the function of these mutants in more detail.

4. Discussion

4.1. Resensitisation rates

In the present study, we examined whether the substitution of Ba^{2+} for Ca^{2+} would affect the rate of resensitisation for the rat neuronal nicotinic α7 receptor. The time constants for the recovery of the 30 μM nicotine response to its control value were found to be 2.2 min in solutions containing 1.8 mM Ca^{2+} and 2.4 min in 1.8 mM Ba^{2+}. These data are in good agreement with the value of 1.9 min found by Fenster et al. (1997) for the rat nicotinic α7 receptor response to 10 μM nicotine. In that case, data from experiments in Ca^{2+} free and Ca^{2+} containing solutions was pooled. Gerzanich et al. (1994) found the time constant for resensitisation of the chick nicotinic α7 receptor in 1.8 mM Ca^{2+} to be 67 s. Here the resensitisation process of nicotinic α7 type receptors is not particularly dependent upon the presence of extracellular Ca^{2+}. This seems to be in contrast to the rat nicotinic α4β2 receptor combination, where the rate of recovery from prolonged applications of nicotine (30 min at 300 nM), is strongly inhibited if extracellular Ca^{2+} is replaced by Ba^{2+} (Fenster et al., 1999).

4.2. Response of wild type nicotinic α7 receptors to agonists

The concentration–response characteristics observed here for the agonist responses of the wild type nicotinic α7 receptor are similar to those found in previous studies. Here, the EC_{50} for the acetylcholine response was 254 μM, (Hill coefficient, 1.3), while the nicotine EC_{50} was 50 μM, (Hill coefficient 1.0). In comparison, Séguéla et al. (1993) obtained an EC_{50} of approximately 30 μM for the action of nicotine on the wild type rat nicotinic α7 receptor expressed in oocytes. In transfected SH-5YSY neuroblastoma cells, Puchacz et al., 1994 obtained a nicotine EC_{50} of 25 μM for rat nicotinic α7 receptors (Hill coefficient 1.3) and an acetylcholine EC_{50} of 150 μM (Hill coefficient 1.3). These values are also similar to those quoted for the chick nicotinic α7 receptor (nicotine EC_{50} of 7.8 μM, Hill coefficient of 1.7 μM, Gerzanich et al., 1994; acetylcholine EC_{50} of 115 μM, Hill coefficient 1.5, Couturier et al., 1990). However because of the problems of desensitisation and channel block, it is to be expected that the top of the concentration–response curves are not

well defined in this and other studies, and so the EC_{50} estimates are probably lower than their true value.

4.3. Effects of the Phe[189]Tyr mutation on the response to agonists

Phenylalanine and tyrosine are both amino acids with aromatic side chains of similar size. However the presence of the free hydroxyl group of tyrosine adds polarity to the aromatic side-chain, and provides the opportunity for tyrosine to interact with ligands in a different way to phenylalanine. In other proteins, this mutation decreases the activation energy of the enzyme streptavidin through alterations in H-bonding affecting oxygen binding (Klumb et al., 1998). The same mutation reduces the catalytic activity of phosphoribosyltransferase (Jardin and Ullman, 1997), and also reduces the K_D of plasminogen activating factor (De Serrano and Castellino, 1994).

In the present study, the concentration–response characteristics produced by the application of acetylcholine to the Phe[189]Tyr mutant were not distinguishable from those obtained for the wild type receptor and the normalised current responses to high concentrations of acetylcholine appeared similar. There seems to be no evidence that the Phe[189]Tyr mutation has altered acetylcholine sensitivity. At first glance it also appears that there is little difference between the responses of the wild type and mutant receptors to nicotine. However when the responses of the mutant to high concentrations of nicotine are compared to the response at 30 μM, they are not significantly increased for the mutant receptor, although they clearly are for the wild type. It would thus appear possible that the mutation reduces the sensitivity of the nicotinic α7 receptor to nicotine, although direct comparison of the peak normalised responses to 500 μM nicotine do not reveal a significant difference ($P > 0.05$) between the wild type and mutant receptors. If the mutation causes a change of efficacy, rather than affinity (assuming there has been no change in the rate of desensitisation), then one might have expected to see a reduction in the peak current response to nicotine, as well as a reduction in the Hill slope (Colquhoun, 1998). The data presented here do not provide firm evidence for such a mechanism. Also, there seems no real reason why a single amino acid cannot be involved in both ligand binding and gating, and therefore in affinity and efficacy. Complete definition of the mechanism by which the sensitivity of the receptors to nicotine and acetylcholine may be altered would require a single channel study of burst kinetics for both agonists at multiple concentrations. Such a detailed and extensive study is beyond the scope of the present work. Nonetheless the present work does not rule out the possibility that the presence of a phenylalanine at position 189 makes some contribution to the responsiveness of the nicotinic α7 receptor to nicotine.

4.4. Antagonism of wild type nicotinic α7 and Phe[189]Tyr mutant receptors by α-bungarotoxin

The sensitivity of the nicotinic α7 receptor and mutant receptor, Phe[189]Tyr, to α-bungarotoxin were assessed by an electrophysiological method. For both receptors, the rate of onset of inhibition appeared to be directly proportional to the toxin concentration. If so, this would be consistent with a simple bimolecular model for the binding of toxin to the receptors. However, although the nicotinic α7 subunit and the mutant subunit differed in one amino acid in the ligand binding site, K_D values for α-bungarotoxin at the nicotinic α7 and mutant Phe[189]Tyr mutant receptors were similar at 4.7 nM and 5.2 nM respectively. These values obtained by an electrophysiological approach are of the same order as the previous K_D values obtained for the chick and human wild type nicotinic α7 receptors using binding studies. The K_D value obtained for the native, human nicotinic α7 receptor was 1.06 nM, whereas α-bungarotoxin at the human nicotinic α7 receptor expressed in oocytes produced a K_D of 0.81 nM (Peng et al., 1994). An α-bungarotoxin binding study provided a similar result ($K_i = 2.4$ nM) at purified chick nicotinic α7 receptors (Gotti et al., 1994). Therefore it would seem that this function-based assay is in good agreement with other approaches for estimating the K_D of this high-affinity toxin.

In this study, the mutation of phenylalanine[189] to tyrosine has not affected the affinity of α-bungarotoxin binding. Among nicotinic receptor subunits generally, the absence of an aromatic residue at position 189 is associated with resistance to α-bungarotoxin binding (Barchan et al., 1995). However as noted above, the α9 subunit is an exception to this. Therefore although the substitution of a tyrosine residue at position 189 can maintain the sensitivity of the receptor to α-bungarotoxin, this result does not prove that an aromatic residue in this position is an absolute requirement for toxin binding in homomeric receptors. However the result does suggest that there has not been widespread disruption of structure–function relationships by the mutation, so that the possible effects on nicotine sensitivity discussed above may be localised to the site of the mutation itself.

4.5. Additional mutations

Preliminary results demonstrated that the mutant Tyr[190]Gln receptor has a markedly decreased responsiveness to acetylcholine and nicotine, only being activated by concentrations of nicotine above 500 μM with a holding potential of −100 mV. This apparant lack of agonist sensitivity prohibited a detailed study of the agonist concentration–response relationship. However if it assumed that the receptor does assemble correctly, the result could imply that the aromatic tyrosine residue at position 190 is

necessary for the maintenance of normal ligand binding in nicotinic α7 receptors. This result is consistent with previous studies on nicotinic α1 receptor subunits which have shown that even the conservative mutation of tyrosine to phenylalanine can radically reduce the sensitivity of the receptor to acetylcholine through alterations in both binding and affinity (Tomaselli et al., 1991; Chen et al., 1995). Hence in both homomeric and heteromeric nicotinic receptors, the conservation of tyrosine at amino acid position 190 seems critical for function.

The glutamate residue at position 261 is thought to lie at the mouth of the ion channel along with other negatively charged amino acids in α1 subunits (Imoto et al., 1988, Dani, 1989, Konno et al., 1991). It seems probable that the glutamate at the equivalent position in the α7 subunit contributes to the putative rings of negatively charged amino acids which are thought to line the wall of the ion channel in heteromeric nicotinic receptors (Imoto et al., 1988). The substitution of the nonpolar residue alanine in place of the acidic residue glutamate would greatly interfere with the negatively charged ring structure mentioned above. It is perhaps as a consequence of this that the mutation could not be studied as no functional expression was detected. In the chick nicotinic α7 subunit M2 domain leucine 247 has also been mutated to a variety of different amino acids. The effects included a general reduction in the rate of desensitisation, increased sensitivity to agonists and an alteration in the pharmacological response to antagonists whereby they became agonists (Revah et al., 1991, Bertrand et al., 1992b). Mutations in putative charge rings at positions lower than that containing glutamate^{261} reduced the Ca^{2+} permeability of nicotinic α7 receptors, but increased the sensitivity to acetylcholine (Bertrand et al., 1993). The result obtained here with the mutant Glu^{261}Ala seems to reduce the sensitivity to acetylcholine, but nonetheless reinforces the prevailing view that the charged ring structure of the nicotinic α7 receptor ion pore region is generally similar to that of heteromeric nicotinic receptors.

The mutation of aspartate^{268} at the apex of the putative M2–M3 extracellular loop examines a region not much studied in the nicotinic α7 homomeric receptor. In contrast to the Glu^{261}Gln mutation, a weak functional response was detectable with the Asp^{268}Ala mutant. The greatly decreased sensitivity of the mutant receptor to acetylcholine and nicotine suggests that the serine–aspartate^{268}–serine, or SDS, motif has functional importance in homomeric receptors. As discussed above, in muscle type receptors, the first extracellular domains of non-α subunits contribute a negative subsite to the pocket in which agonists bind. Unless stereoisomers of nicotinic α7 subunits are produced in the oocyte, it seems unlikely that the first extracellular domains of two adjacent nicotinic α7 subunits will be closely aligned in a homomeric receptor. However there is presumably still a requirement for a negative subsite in the ligand binding region. It therefore seems possible that the negative SDS domain, which is conserved in homomeric nicotinic α subunits, could fulfil the function performed by the aspartate residues of non-α subunits in heteromeric receptors. In this situation, although the general charge arrangement of the binding site would be conserved, the amino acid domains supplying those charged residues could be markedly different for homomeric receptors. In future studies, it will be interesting to investigate the effects of altering size, charge and polarity of the amino acids comprising the SDS motif.

Overall, the results obtained here suggest that tyrosine is as effective as phenylalanine in anchoring α-bungarotoxin to a negatively charged binding pocket (Dougherty, 1996) and mediating the response of the nicotinic α7 receptor to acetylcholine. However tyrosine may not be able to perfectly substitute for phenylalanine in providing the nicotinic α7 subunit with its high sensitivity to nicotine. The α-bungarotoxin binding result suggests that the effects of the Phe^{189}Tyr mutation are quite localised and do not greatly disrupt receptor structure. In common with heteromeric nicotinic receptors, the amino acids tyrosine^{190} and glutamate^{261} seem critical for receptor function. This is consistent with the idea that the gross architecture of the first extracellular domain and the M2 domain are similar between homomeric α7 and heteromeric α1–α4 containing nicotinic receptors. However the loss of function resulting from the mutation of aspartate^{268} to alanine in the second extracellular domain is consistent with the idea that this region could supply an anionic domain to the ligand binding site. Therefore, although the essential form of the agonist binding site seems to be maintained in all nicotinic receptors, some aspects of the structure–function relationships contributing to it could be substantially different for homomeric receptors.

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