Volume 332, number 1,2, 139–142 FEBS 13087 October 1993

Residues 1 to 80 of the N-terminal domain of the β subunit confer neuronal bungarotoxin sensitivity and agonist selectivity on neuronal nicotinic receptors

Susan V. Wheeler**, John E. Chad, Richard Foreman*

Department of Physiology and Pharmacology, University of Southampton, Bassett Crescent East, Southampton, SO9 3TU, UK

Received 19 July 1993

Standard two electrode voltage clamp techniques were used to investigate the response of neuronal nicotinic acetylcholine receptors, expressed in *Xenopus* oocytes, to various agonists and neuronal bungarotoxin (NBT). The β subunit is an important determinant of the receptor's pharmacological profile. Co-expression of α4 and β2 subunits produced a receptor that was relatively insensitive to cytisine and nicotine and inhibited by NBT, whilst the α4β4 combination produced a receptor that was highly sensitive to cytisine and nicotine but resistant to toxin. The first 80 amino acids of the N-terminal domain of the β subunit are implicated in these characteristics, since the combination of α4 with a hybrid β subunit comprising amino acids 1 → 80 of β2 and 81 → 416 of β4 became relatively insensitive to nicotine and cytisine and resistant to inhibition by neuronal bungarotoxin.

Nicotinic receptor; Chimeric subunit; *Xenopus* oocyte; Neuronal bungarotoxin

1. INTRODUCTION

The functional diversity of nicotinic acetylcholine receptors (nAChRs) in the mammalian central nervous system is increased by the occurrence of multiple α and β subunits which may be expressed in different pairwise combinations in distinct sets of neurons in particular regions of the brain [1] and at varying stages during development of the nervous system [2]. The differing pharmacological properties of these receptors have been examined by their expression in *Xenopus* oocytes (reviewed in [3]). Neuronal nAChRS are pentameric structures, analogous to those found in muscle, and are comprised of two α and three β subunits [4,5]. Both types of subunit contribute to the agonist binding [6], toxin sensitivity [7,8] and channel characteristics [9–11] of the resulting receptor. For example, neuronal receptors containing a β2 type subunit are sensitive to inhibition by neuronal bungarotoxin [NBT] and resistant to the actions of the nicotinic agonist cytisine, whilst a combination of the same α subunit with β4 produces a receptor that is insensitive to NBT but stimulated by cytisine. Here we report the production of a β2/β4 chimeric subunit consisting of the amino-terminal 80 residues of the β2 subunit (approximately 38% of the extracellular domain) joined to the appropriate remainder of the β4 sequence. The construct was co-expressed in oocytes with the neuronal α4 subunit and the pharmacological properties of the resulting receptor assessed in an effort to determine the relative contribution made by the amino-terminal region of the β sequence.

*Corresponding author. Fax: (44) (703) 594 319.

***Present address:* Department of Biochemistry, University of Bath, Bath, BA2 7AY, UK.

2. MATERIALS AND METHODS

DNA and RNA modifying enzymes were supplied by Promega or Boehringer. All other reagents were from Sigma and were Analar grade or better. Neuronal bungarotoxin was supplied by Biotoxins Inc. General methods for restriction digests, fragment purification and ligation have been described elsewhere [11]. The β2/β4 chimeric subunit was constructed by cleavage of β4 cDNA in the vector pBluescript SK(−) (Stratagene Ltd., Cambridge) with *Bam*HI and *Not*I to remove a 403 bp fragment which encoded the 5′ untranslated sequence, the signal peptide and residues 1 to 80 of the mature β4 protein. A PCR [12] method was used to introduce a *Bam*HI site in the analogous position in the β2 sequence (a single C to A change at nucleotide 244) and the corresponding 5′ fragment of the β2 sequence was excised and purified. This fragment was ligated into the *Bam*HI/*Not*I cleaved β4 containing vector.

Messenger RNAs coding for the α3, β2, β4 and chimeric subunits were prepared by in vitro transcription of full-length linearised cDNAs as previously described [13]. Transcripts were stored as ethanolic precipitates at −70°C. For injection transcripts were vortexed to produce a homogeneous mix, and a 5 μg aliquot of the required subunits removed, mixed and then centrifuged. Precipitates were washed in 70% (v/v) ethanol, dried then resuspended in ribonuclease free diethylpyrocarbonate treated water. Approximately 15 ng of each subunit was coinjected into individual oocytes in the required combinations in a total volume of 30 to 50 nl.

Oocytes were removed from anaesthetised *Xenopus* and treated with 2 mg · ml^{-1} collagenase (Sigma type II) at 18–20°C for 50 min followed by manual removal of the follicular layers using watchmakers forceps. Injected oocytes were maintained at ≈18°C, for 2 to 5 days in changes

of SOS medium (100 mM NaCl, 2 mM KCl, 1.8 mM $CaCl_2$, 1 mM $MgCl_2$, 5 mM HEPES, pH 7.6). For electrophysiological recording oocytes were held in a 1 ml perspex bath and impaled with two microelectrodes filled with 3 M KCl, pH 7.8. Both electrodes had resistances of less than 1 MΩ. Oocytes were superfused with SOS plus 1 μM atropine at 12 ml · min^{-1} and clamped at −80 mV unless otherwise stated. The desired dose of agonist was added to perfusing SOS and applied for 5 s. To study the effect of NBT oocytes were incubated with the desired concentration of neurotoxin for 15 min prior to wash out and immediate application of agonist. Data acquisition, storage and analysis were performed using a Dell System 200 PC AT using the programme pCLAMP. Peak current dose–response curves from 10^{-8} M to 10^{-3} M were constructed for each agonist and data normalised to a non-desensitising dose in the mid range of the curve (10 μM) for each oocyte.

3. RESULTS AND DISCUSSION

Four ganglionic agonists, acetylcholine (ACh), 1,1-dimethyl-4-phenylpiperazinium (DMPP), nicotine (Nic) and cytisine (Cyt) were applied to oocytes expressing $\beta 2$, $\beta 4$ or $\beta 2/4$ chimera in combination with $\alpha 4$. In all cases oocytes expressing neuronal subunits exhibited dose dependent inward currents upon application of these agonists under voltage clamp. Fig. 1 shows the full dose–response curves for these agonists with the three receptor combinations under consideration. In order to examine the relative potency of these agonists, where absolute current values differed, a comparison was made with an equivalent dose (10 μM) of acetylcholine and these ratios are shown in Fig. 2. In common with others [6], we found that cytisine was the most potent agonist for $\alpha 4\beta 4$ receptors ($\times$ 5.68 an equivalent dose of ACh, $n = 4$) but a poor stimulant of $\alpha 4\beta 2$ ($\times$ 0.29 the equivalent ACh dose, $n = 4$). Replacement of the first 80 residues of the $\beta 4$ polypeptide chain with the corresponding region of $\beta 2$ resulted in a substantial decrease in the response to cytisine when applied to oocytes expressing the chimeric subunit ($\times$ 0.62 an equivalent ACh dose, $n = 5$). The conferment of $\beta 2$-like properties was also seen in the response to nicotine. The potency of nicotine as an agonist relative to ACh is similar for subunits containing $\beta 2$ or the chimera ($\times$ 1.0 for $\beta 2$ and $\times$ 1.13 for the chimera, $n = 4$ in both cases) when compared to $\beta 4$ ($\times$ 5.4, $n = 3$). The response of the chimera to the ganglionic agonist DMPP ($\times$ 0.4 the ACh dose) was intermediate with those shown by $\alpha 4\beta 4$ ($\times$ 0.13 the ACh dose) and $\alpha 4\beta 2$ ($\times$ 0.68 the ACh dose) combinations. The potency of DMPP may reflect the relative contribution to agonist binding of more distal regions of the β chain which have remained unchanged in the chimera, nevertheless the experiments presented here suggest that regions within the first 80 residues of the β chain can exert a major influence on agonist sensitivity. In a recent study Figl et al. [14] also demonstrated that a region towards the centre of the extracellular domain of the $\beta 4$ subunit was a major determinant in conferring agonist sensitivity when β chimeras were co-expressed with $\alpha 3$. Their localisation of this determinant to residues 105 to 115, rather than earlier in the sequence, as our experiments indicate, may arise from differences in the nature of β chimeras or in the requirements of the differing α subunits used for oocyte expression. $\alpha 3$ and $\alpha 4$ expressed in oocytes with the same β subunit show differing agonist sensitivities and rates of desensitisation [15]. Similar distinctions may also extend to the nature and consequences of α/β interactions.

A minor component of *Bungarus multicinctus* venom, known as neuronal bungarotoxin (NBT), blocks neuronal nicotinic receptors in the concentration range 10–100 nM but has relatively little effect, at similar concentrations, on receptors found at the neuromuscular junction [16–18]. The sensitivity of neuronal receptors to NBT is another property which varies according to subunit composition and Fig. 3 shows the contribution of the β subunit towards susceptibility to toxin inhibition. Receptors composed of $\alpha 4\beta 2/4$ do not differ significantly from $\alpha 4\beta 2$ in their response to 10 μM acetylcholine following treatment with 100 nM NBT, implying that at least some determinants for toxin binding reside in the amino-terminal region of the β polypeptide chain.

Studies using a variety of crosslinking reagents [19] and synthetic peptides [20] have defined those regions of the α subunit which are involved in the binding of agonists and toxins to the extracellular domain of the assembled receptor. At present no such information is available for the β subunit, although expression of tran-

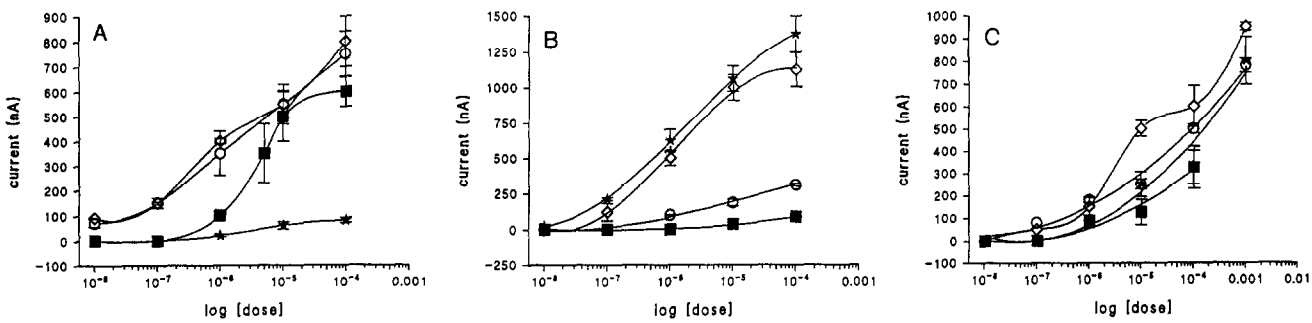

Fig. 1. Agonist-induced current responses for oocytes expressing the receptor subunit combinations $\alpha 4\beta 2$ (A), $\alpha 4\beta 4$ (B) and $\alpha 4\beta 2/4$ (C). Currents were elicited by bath application of various concentrations of cytisine (★), DMPP (■), nicotine (◇) and acetylcholine (○).

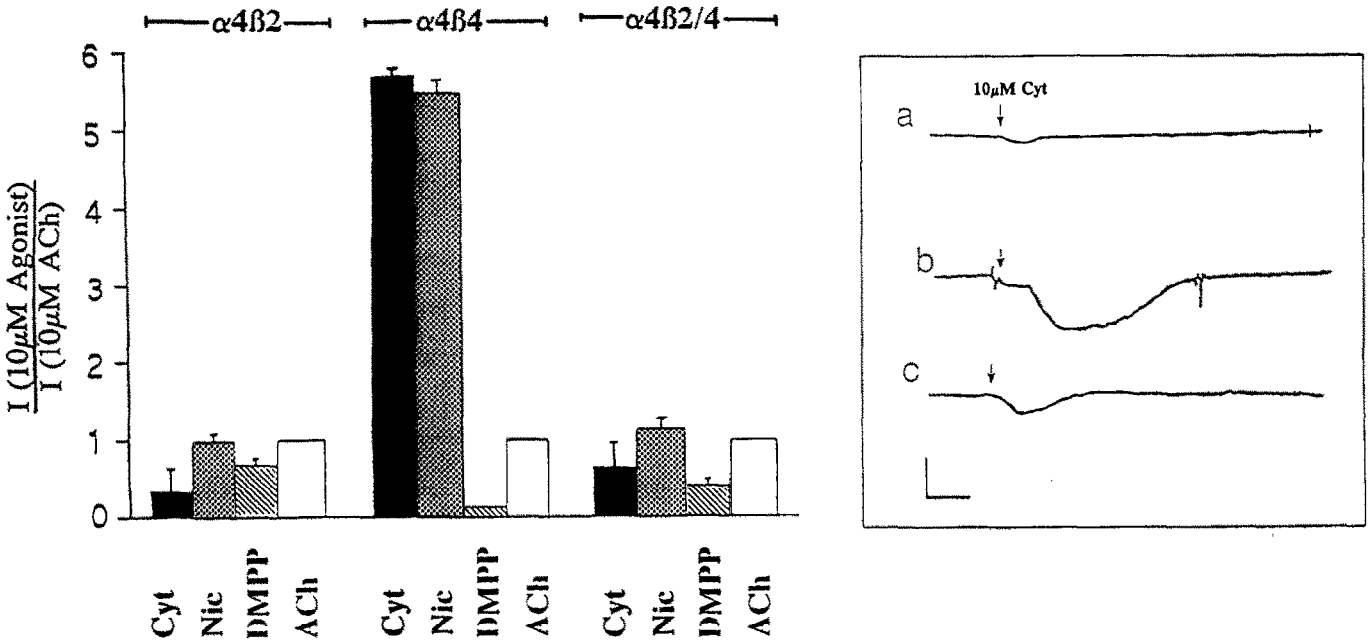

Fig. 2. Ratio of the currents induced by the application of 10 μM of various agonists to that obtained from an identical dose of acetylcholine (the ratio for ACh is therefore 1). Each column represents the mean ± S.E.M. of the ratios of 3–5 separate oocytes from two different animals. The boxed portion of the figure shows typical responses of the three chimeric receptors to the application of 10 μM cytisine; a = $\alpha 4\beta 2$, b = $\alpha 4\beta 4$, c = $\alpha 4\beta 2/4$. The scale bars in the bottom left of the box represent 250 nA (vertical axis) and 5 s (horizontal axis).

scripts in *Xenopus* oocytes [6,8] has indicated the modifying nature of this constituent on receptor pharmacology. One possibility is that the ligand binding site of neuronal nAChRs is found at the interface of the α and β chains and is comprised of parts of both subunits. Alternatively, the β subunit may act to modulate binding by the α subunit via allosteric interactions involving regions of the polypeptide chain or attached carbohydrate groups. Here we show that, whatever the mechanism involved, these characteristics of the β subunit are determined by the first 80 residues of the polypeptide although we cannot identify as yet which determinants within this region are of primary importance.

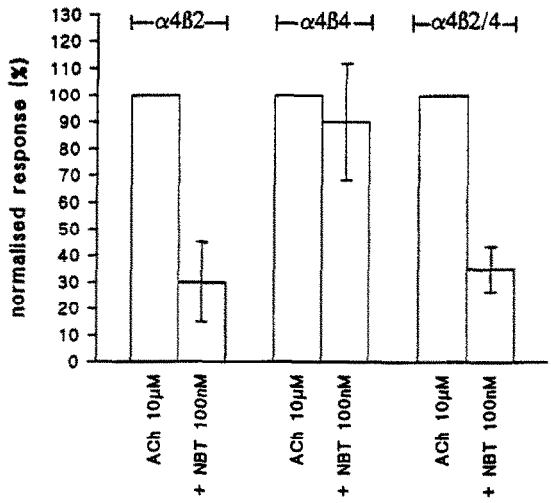

Fig. 3. The action of 100 nM neuronal bungarotoxin (NBT) on oocytes expressing $\alpha 4\beta 2$ ($n = 4$), $\alpha 4\beta 2/4$ ($n = 10$) and $\alpha 4\beta 4$ ($n = 3$) receptors. Responses are presented after normalisation to the response of the same oocyte to 10 μM acetylcholine (response to 10 μM ACh = 100) and are shown ± S.E.M.

Putative asparagine-linked glycosylation sites in the extracellular domain are a common feature of all neuronal nicotinic genes sequenced thus far (for a review see [21]). Fig. 4 shows the glycosylation sites of the $\beta 2$ and $\beta 4$ amino-terminal regions together with those preserved in the $\beta 2/4$ construct. The binding of a large molecule such as a peptide toxin may be influenced by the cluster of branched oligosaccharide groups located between residues 72 and 145 in $\beta 4$. Thus, the removal of one of these oligosaccharide groups by abolition of the attachment site at position 72 may serve to increase

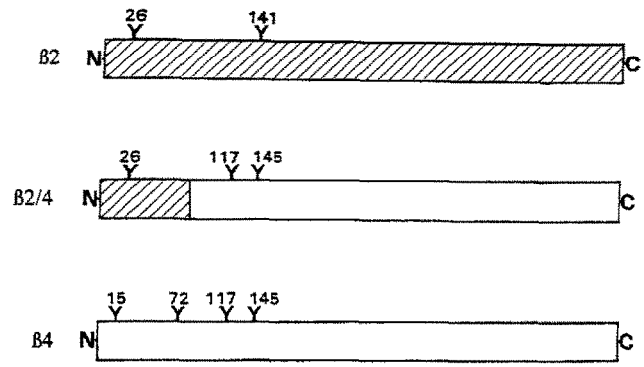

Fig. 4. Sites of oligosaccharide attachment in the amino-terminal extracellular domains of the $\beta 2$ and $\beta 4$ subunits and the chimeric $\beta 2/4$ construct.

the sensitivity of the chimera to inhibition by NBT. It is possible that the combined degree of glycosylation of both α and β subunits may contribute toward the differing sensitivities to NBT shown by various subunit combinations. For example, $\beta 2$ in combination with $\alpha 3$ or $\alpha 4$ (both with two glycosylation sites) gives a receptor inhibited by pre-incubation with 100 nM NBT, in contrast to $\alpha 2\beta 2$ ($\alpha 2$ has three glycosylation sites) which is NBT-resistant at this concentration [8,21]. In combination with the highly glycosylated $\beta 4$ subunit however, both $\alpha 3$ and $\alpha 4$ are completely insensitive to NBT when pre-incubated with the toxin ([23] and this report). The influence of attached oligosaccharide groups may arise as a result of direct steric hindrance to the access of toxin or from a more subtle effect on the kinetics of toxin binding. In support of the latter proposal, Papke et al. [24] have recently shown that $\alpha 3\beta 4$ receptors are inhibited by NBT if the toxin is co-applied with agonist rather than administered by pre-incubation. The inference here is that subunit combination dictates the kinetics of toxin binding, and glycosylation may be one factor in determining the relative association and dissociation rates for this process. In agreement with our findings, these authors also demonstrate a direct relationship between the origin of the amino terminal 130 residues ($\beta 2$ or $\beta 4$) and sensitivity to inhibition by neurotoxin.

The experiments reported here identify a region of the nicotinic neuronal β subunit which mediates two important functional properties of the receptor, response to different agonists and inhibition by neurotoxin. The significance of post translational modifications and/or individual amino acids within this sequence can be established by oligonucleotide directed mutagenesis and is the subject of further investigation.

Acknowledgements: We are grateful to Dr S. Heinemann (Salk Institute) for the $\alpha 4$ and $\beta 2$ cDNAs and to Dr J. Connolly (University of Strathclyde) for $\beta 4$. We also thank Dr Lindy Holden-Dye for her helpful comments and the SERC, The Wessex Medical Trust and The Sir Halley Stewart Trust whose grants supported this work.

REFERENCES

[1] Wada, E., Wada, K., Boulter, J., Deneris, E.S. and Heinemann, S. (1989) J. Comp. Neurol. 284, 314–335.
[2] Moss, B.L., Schuetze, S.M. and Role, L.W. (1989) Neuron 3, 597–607.
[3] Deneris, E.S., Connolly, J., Rogers, S.W. and Duvoisin, R. (1991) Trends Pharmacol. Sci. 12, 34–40.
[4] Anand, R., Conroy, W.G., Schoepfer, R., Whiting, P. and Lindstrom, J. (1991) J. Biol. Chem. 266, 11192–11198.
[5] Cooper, E., Couturier, S. and Ballivet, M. (1991) Nature 350, 235–238.
[6] Luetje, C.W. and Patrick, J. (1991) J. Neurosci. 11, 837–845.
[7] Deneris, E.S., Boulter, J., Swanson, L., Patrick, J. and Heinemann, S. (1989) J. Biol. Chem. 264, 6268–6272.
[8] Luetje, C.W., Wada, K., Rogers, S., Abramson, S.N., Tsuji, K., Heinemann, S. and Patrick, J. (1990) J. Neurochem. 55, 632–640.
[9] Papke, R.L., Boulter, J., Patrick, J. and Heinemann, S. (1989) Neuron 3, 589–596.
[10] Papke, R.L. and Heinemann S. (1991) J. Physiol. 440, 95–112.
[11] Charnet, P., Labarca, C., Cohen, B.N., Davidson, N., Lester, H.A. and Pilar, G. (1992) J. Physiol. 450, 375–394.
[12] Higuchi, R., Krummel, B. and Saiki, R.K. (1988) Nucleic Acids Res. 16, 7351–7367.
[13] Boulter, J., Evans, K.L., Gardner, P.D., Goldman, D., Heinemann, S., Martin, G., Mason, G., Patrick, J. and Treco, D. (1986) in: Molecular Aspects of Neurobiology (R. Levi Montalcini et al., Eds.) Springer, Berlin.
[14] Figl, A., Cohen, B.N., Quick, M.W., Davidson, N. and Lester, H.A. (1992) FEBS Lett. 308, 245–248.
[15] Gross, A., Ballivet, M., Rungger, D. and Bertrand, D. (1991) Pflügers Arch. 419, 545–551.
[16] Ravdin, P.M. and Berg, D.K. (1979) Proc. Natl. Acad. Sci. USA 76, 2072–2076.
[17] Chiappinelli, V.A. (1983) Brain Res. 277 9–21.
[18] Loring, R.H., Chiappinelli, V.A., Zigmond, R.E. and Cohen, J.B. (1984) Neuroscience 11, 989–999.
[19] Kao, P.N., Dwork, A.J., Kaldany, R-R.J., Silver, M.S., Wideman, J., Stein, S. and Karlin, A. (1984) J. Biol. Chem. 259, 11662–11665.
[20] Conti-Tronconi, B.M., Fels, G., McLane, K., Tang, F., Bellone, M., Kokla, A., Tzartos, S., Milius, R. and Maelicke, A. (1989) in: Molecular Neurobiology of Neuroreceptors and Ion Channels (Maelicke, A., Ed.) NATO-ASI, series H, Vol. 32, pp. 291–310, Springer, Berlin.
[21] Sargent, P.B. (1993) Annu. Rev. Neurosci. 16, 403–443.
[22] Boulter, J., Connelly, J., Deneris, E., Goldman, D., Heinemann, S. and Patrick, J. (1987) Proc. Natl. Acad. Sci. USA 84, 7763–7767.
[23] Duvoisin, R.M., Deneris, E.S., Patrick, J. and Heinemann, S. (1989) Neuron 3, 487- 496.
[24] Papke, R.L., Duvoisin, R.M. and Heinemann, S.F. (1993) Proc. R. Soc. Lond. B. 252, 141–148.