ELSEVIER

European Journal of Pharmacology 357 (1998) 83–92

ejp

The actions of muscle relaxants at nicotinic acetylcholine receptor isoforms

Catherine M. Garland, Richard C. Foreman, John E. Chad, Lindy Holden-Dye *, Robert J. Walker

Pharmacology Group, Division of Cell Sciences, University of Southampton, Bassett Crescent East, Southampton SO16 7PX, UK

Received 7 July 1998; accepted 17 July 1998

Abstract

The pharmacological diversity of the different isoforms of the nicotinic acetylcholine receptor arises from the diversity of the subunits that assemble to form the native receptors. The aim of this study was to investigate the actions of the muscle relaxants d-tubocurarine, pancuronium and vecuronium on different isoforms of nicotinic acetylcholine receptors (mouse foetal muscle, mouse adult muscle and a rat neuronal), using the *Xenopus* oocyte expression system. Oocytes were injected with cRNAs for α, β, γ, δ subunits (the native foetal muscle subunit combination), or with cRNAs for α, β, ε, δ subunits (the native adult muscle subunit combination), or with cRNAs for α4β2 subunits (a putative native neuronal subunit combination). Acetylcholine had a similar potency at all three subunit combinations (EC_{50} 11.6, 17.4 and 19.1 μM, respectively). At all three receptor types, d-tubocurarine and pancuronium blocked the responses elicited by acetylcholine in a reversible manner. Furthermore, the inhibition of the acetylcholine currents for the foetal and adult nicotinic acetylcholine receptor by pancuronium and d-tubocurarine was independent of the holding voltage over the range −100 to −40 mV. In oocytes expressing the foetal muscle nicotinic acetylcholine receptors the inhibition of the current in response to 100 μM acetylcholine by 10 nM d-tubocurarine was 29 ± 5% (mean ± S.E.M.; $n = 7$), and the inhibition by 10 nM pancuronium was 39 ± 6% (mean ± S.E.M.; $n = 8$; $P > 0.05$ vs. d-tubocurarine). However, in the adult form of the muscle nicotinic acetylcholine receptor, 10 nM d-tubocurarine and 10 nM pancuronium were both more effective at blocking the response to 100 μM acetylcholine compared to the foetal muscle nicotinic acetylcholine receptor, with values of 55 ± 5% ($P < 0.01$; $n = 12$) and 60 ± 4% ($P < 0.001$; $n = 10$), respectively. Thus the developmental switch from the γ to the ε subunit alters the antagonism of the nicotinic acetylcholine receptor for both pancuronium and d-tubocurarine. Vecuronium was more potent than pancuronium. One nM vecuronium reduced the response to 100 μM acetylcholine by 71 ± 6% ($n = 10$) for foetal and 63 ± 5% ($n = 4$) for adult nicotinic acetylcholine receptors. In the α4β2 neuronal nicotinic acetylcholine receptor combination, 10 nM pancuronium was a more effective antagonist of the response to 100 μM acetylcholine (69 ± 6%, $n = 6$) than 10 nM d-tubocurarine (30 ± 5%; $n = 6$; $P < 0.05$ compared to pancuronium). This is in contrast to the adult muscle nicotinic acetylcholine receptor, where pancuronium and d-tubocurarine were equieffective. The expression of the β2 subunit with muscle α, ε and δ subunits formed a functional receptor which was blocked by pancuronium and d-tubocurarine in a similar manner to the αβ1εδ subunit consistent with the hypothesis that the β subunit is not a major determinant in the action of this drug at the adult muscle nicotinic acetylcholine receptor.

Keywords: Nicotinic acetylcholine receptor; Muscle relaxant; d-Tubocurarine; Pancuronium; *Xenopus* oocyte; Voltage-clamp; Two-electrode

1. Introduction

Clinically used aminosteroid muscle relaxants pancuronium and vecuronium (Fig. 1) are known to block neuromuscular transmission in a similar manner to d-tubocurarine (Buckett, 1968a,b; Savage et al., 1980). In vivo, vecuronium is a more potent muscle relaxant and has a shorter duration of action (Savage et al., 1980; Fahey et al., 1981) than pancuronium, which in turn is more potent than d-tubocurarine (Baird and Reid, 1967). The site of action of these muscle relaxants is the nicotinic acetylcholine receptor, located at the muscle endplate. Briefly, this receptor is a pentamer, composed of four different subunits, 2α, β, γ and δ, in foetal muscle (Reynolds and Karlin, 1978). In the adult muscle, the γ subunit is substi-

* Corresponding author. Tel.: +44-1703-594295; Fax: +44-1703-594319; E-mail: lmhd@soton.ac.uk

0014-2999/98/$19.00
PII: S0014-2999(98)00542-1

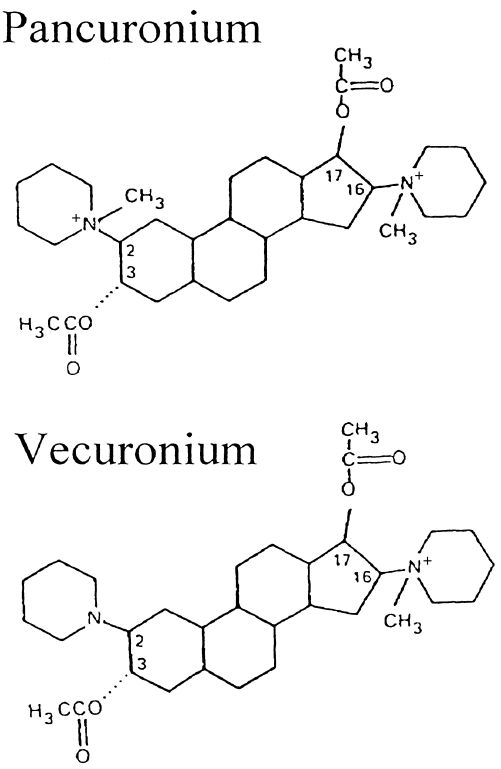

Fig. 1. Structure of vecuronium and pancuronium.

tuted by an ε subunit (Mishina et al., 1986; Schuetze and Role, 1987). The α subunits are characterised by the presence of two vicinal cysteines (Noda et al., 1982) at positions 192 and 193. This region contributes to the ligand binding site for acetylcholine (Kao et al., 1984; Neumann et al., 1985; Neumann et al., 1986).

A more detailed analysis of the binding sites for acetylcholine and muscle relaxants to the muscle nicotinic acetylcholine receptor has been carried out at the molecular level. The muscle nicotinic acetylcholine receptor has two agonist binding sites which are dissimilar, having different affinities for acetylcholine (Weber and Changeux, 1974; Sine et al., 1990). Expression of subunits in different combinations in *Xenopus* oocytes revealed that the association of an α subunit with either a γ or a δ subunit was necessary for agonist binding (Kurosaki et al., 1987) and the acetylcholine binding sites are located at the interfaces of the α/γ and the α/δ subunits (Sine et al., 1990; Sine and Claudio, 1991; Sine, 1993).

The regions of the α subunit that contribute to the binding of d-tubocurarine and curare-like antagonists are three conserved tyrosines (Tyr^{93}, Tyr^{190} and Tyr^{198}) near the proposed ligand binding pocket of the nicotinic acetylcholine receptor (Sine et al., 1994). Substitution of Tyr^{198} to a phenylalanine leads to a decreased affinity for acetylcholine (Tomaselli et al., 1991; O'Leary et al., 1994), but an increase in affinity for d-tubocurarine. Notably, this enhancement was selective for d-tubocurarine and was not evident for other muscle relaxants such as gallamine and pancuronium (Filatov et al., 1993). This indicates that although the mode of action for these muscle relaxants may be similar, at least some of the differences seen between pancuronium and d-tubocurarine in vivo may be due to differential binding of the antagonist molecules within the characterised ligand binding site of the nicotinic acetylcholine receptor.

In addition to the integral role of the α subunits in the binding of acetylcholine and muscle relaxants, it has been shown that other nicotinic receptor subunits can also influence ligand binding for both agonists and antagonists. For example, in the native nicotinic acetylcholine receptor, the two α binding sites are nonidentical, and show differing affinities for d-tubocurarine (Neubig and Cohen, 1979). One α subunit together with the γ subunit comprise a high affinity acetylcholine binding site, while the second α subunit together with the δ subunit forms the other, lower affinity binding site (Blount and Merlie, 1989; Pederson and Cohen, 1990). As the two α subunits are identical, this heterogeneity of the binding sites must be due to the differences between the γ and δ subunit.

As well as being found in skeletal muscle, nicotinic acetylcholine receptors are also present on autonomic neurones and adrenal chromaffin cells in the peripheral nervous system and on many neurones found in the central nervous system (CNS). To date, eight neuronal α subunits (α2 to α9) and three β subunits (β2 to β4; Mauron et al., 1985; Boulter et al., 1986; Goldman et al., 1987; Gerzanich et al., 1993; Elgoyhen et al., 1994) have been isolated. In the mature mammalian brain, the predominant nicotinic acetylcholine receptor subtype that does not bind α-bungarotoxin is composed of α4 and β2 subunits (Whiting and Lindstrom, 1987, 1988). In neuronal nicotinic acetylcholine receptors it seems plausible that the ligand binding sites are formed in a similar manner to the muscle nictonic acetylcholine receptor, i.e., between the interfaces of the α and β subunits, as both subunits have been shown to influence the pharmacology of the receptor (Deneris et al., 1988; Luetje et al., 1990; reviewed by Luetje and Patrick, 1991; Deneris et al., 1991; Papke and Heinemann, 1993; Harvey et al., 1996; Harvey and Luetje, 1996).

The aims of the study described here were twofold. Firstly, to compare the action of the muscle relaxants at the adult muscle nicotinic acetylcholine receptor with their action at the foetal isoform (differing only in one subunit, ε to γ), and the predominant neuronal isoform (α4β2) of the nicotinic acetylcholine receptor. Secondly, to make a direct comparison between the action of the muscle relaxants d-tubocurarine, pancuronium and vecuronium (Fig. 1) at the adult isoform of the muscle nicotinic acetylcholine receptor. These studies identified differences between the action of d-tubocurarine and pancuronium at nicotinic acetylcholine receptor subtypes and highlighted the marked potency of vecuronium, in comparison to d-tubocurarine and pancuronium, at muscle nicotinic acetylcholine receptor.

2. Materials and methods

2.1. In vitro synthesis of nicotinic acetylcholine receptor mRNA

cDNAs for the muscle subunits α, β, γ and δ, subunits were provided by Professor S. Heinemann, for the neuronal subunits by Professor J. Lindstrom and for the ε subunit by Dr. P. Gardner. All muscle subunits were mouse cDNAs, while the neuronal subunits were from rat. The cDNA was linearised using the appropriate restriction enzymes, and the DNA purified by phenol extraction. This DNA was transcribed in vitro using the SP6 polymerase transcription kit, 'Promega RiboMAX' (Promega, Southampton, UK) according to the manufacturer's instructions. Transcripts were capped at the 5″ end by inclusion of the nucleotide $m^7G(5')ppp(5')G$ (New England Biolabs) in the transcription reaction at a final concentration of 3 mM. This modification has been shown to increase the stability and efficiency of translation of cRNAs in oocytes (Swanson and Folander, 1992). After transcription, the mRNAs were stored as ethanolic precipitates at −70°C until required.

2.2. Expression in Xenopus oocytes

Oocytes were removed by biopsy from mature female *Xenopus laevis* under anaesthesia, 0.15% 3-aminobenzoic acid ethyl ester (Tricaine, Sigma, Poole, UK). The follicular cell layer of the oocyte was removed by digestion with collagenase (type IA, Sigma, 2 mg ml^{-1} in Ca^{2+} free standard oocyte saline (SOS: NaCl 82, KCl 2.7, $MgCl_2$ 1, HEPES 5 mM, pH = 7.6)) at 18°C with slow rotation (10 rpm) for 17 min, followed by manual dissection.

Stage V and VI oocytes were selected and injected with 5–10 ng of each subunit dissolved in diethylpyrocarbonate-treated autoclaved water. The oocytes were then incubated at 18°C in complete SOS (NaCl 100, $CaCl_2$ 1.8, KCl 2, $MgCl_2$ 1, HEPES 5 mM, pH = 7.6) for 24–48 h (muscle types), 48–72 h (neuronal types).

During electrophysiological recordings, oocytes were continually superfused at 6 ml min^{-1} using a gravity driven perfusion system. All drugs were applied via this system. Currents elicited by application of acetylcholine (and acetylcholine plus antagonist) were measured using standard two-electrode voltage clamp technique (GeneClamp 500, Axon Instruments USA). Electrodes were filled with 3 M KCl, the current passing electrode had a resistance of 1–3 MΩ, while the voltage recording electrode had a resistance of 3–5 MΩ. Acetylcholine applications in both the presence and absence of antagonist were for 30 s. To determine the effect of an antagonist on the acetylcholine-elicited response, the saline containing antagonist was perfused on the oocytes for 3 min prior to application of acetylcholine in the presence of the antagonist. Application of antagonist containing SOS were bracketed by the ones which contained acetylcholine only to maintain ongoing measurements of the control response to acetylcholine, throughout the experiment. If the control response changed by more than 10% during the experiment, the data were discarded.

Control experiments of vehicle addition alone produced no changes. Similarly, no change in holding current was observed during perfusion of SOS alone. All experiments were carried out at room temperature (16 to 20°C) and care was taken that the pH of all solutions administered to the oocyte was 7.6.

Results are expressed as the mean ± S.E.M. and significance was tested using the unpaired two-tailed Student's *t*-test (unless otherwise stated). Significance was assumed for $P < 0.05$. Concentration–response curves were fitted to the logistic equation using nonlinear regression analysis to estimate EC_{50} values and maximum currents (given

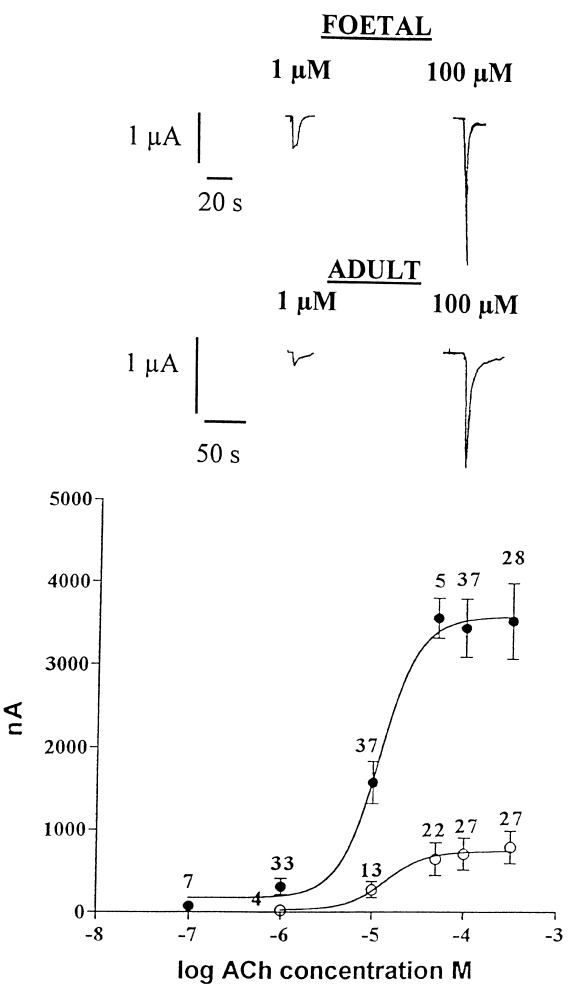

Fig. 2. Acetylcholine (ACh) elicits concentration-dependent inward currents in *Xenopus* oocytes expressing adult and foetal muscle-type nicotinic acetylcholine receptors. Oocytes were voltage clamped at −80 mV. (Traces) Examples of recordings from oocytes expressing foetal (top trace) and adult (lower trace) muscle nicotinic acetylcholine receptors. (Graph) Concentration–response curves for the action of acetylcholine on foetal (●) and adult (○) nicotinic acetylcholine receptors (mean ± S.E.M., *n* as indicated).

with 95% confidence limits) using GraphPad Prism (version 2, San Diego, CA, USA). For inhibition experiments the current responses were normalized to the response to 100 μM acetylcholine.

Pancuronium and vecuronium were obtained from Organon Laboratories (Newhouse, UK). Acetylcholine chloride and d-tubocurarine were obtained from Sigma.

3. Results

3.1. The effect of pancuronium and d-tubocurarine on mouse muscle nicotinic acetylcholine receptors

Acetylcholine was applied (for 30 s) to oocytes, voltage clamped at −80 mV, expressing either αβεδ (adult muscle) or αβγδ (foetal muscle), at concentrations ranging from 1 to 300 μM (adult) and from 1 to 500 μM (foetal). Acetylcholine elicited concentration-dependent inward currents (Fig. 2, traces) in both cases. The data were fitted to the logistic equation (Fig. 2, graph; Hill slope = 2.0; R^2 = 0.99 for the foetal nicotinic acetylcholine receptor currents and $R^2 = 0.988$ for the adult nicotinic acetylcholine receptor currents). The maximal current was greater for the foetal muscle compared to the adult muscle nicotinic acetylcholine receptor, 3881 nA (3032 to 4730; $n = 21$) compared to 845 nA (741 to 949; $n = 31$). EC_{50} values were similar, 11.6 μM (3.2 to 41; $n = 21$) for foetal muscle nicotinic acetylcholine receptor and 17.4 μM (8.7 to 34; $n = 31$) for adult muscle nicotinic acetylcholine receptor.

Application of 10 nM pancuronium and 10 nM d-tubocurarine alone, on both muscle isoforms of the nicotinic acetylcholine receptor, did not give rise to any current. However, both pancuronium and d-tubocurarine decreased the size of the acetylcholine elicited current (Fig. 3). The percentage inhibition of the response to 100 μM acetylcholine by 10 nM d-tubocurarine and 10 nM pancuronium was determined for both receptors. In oocytes expressing the foetal muscle nicotinic acetylcholine receptors the inhibition by 10 nM d-tubocurarine was 29 ± 5% ($n = 7$), whereas the inhibition by 10 nM pancuronium was 39 ± 6%

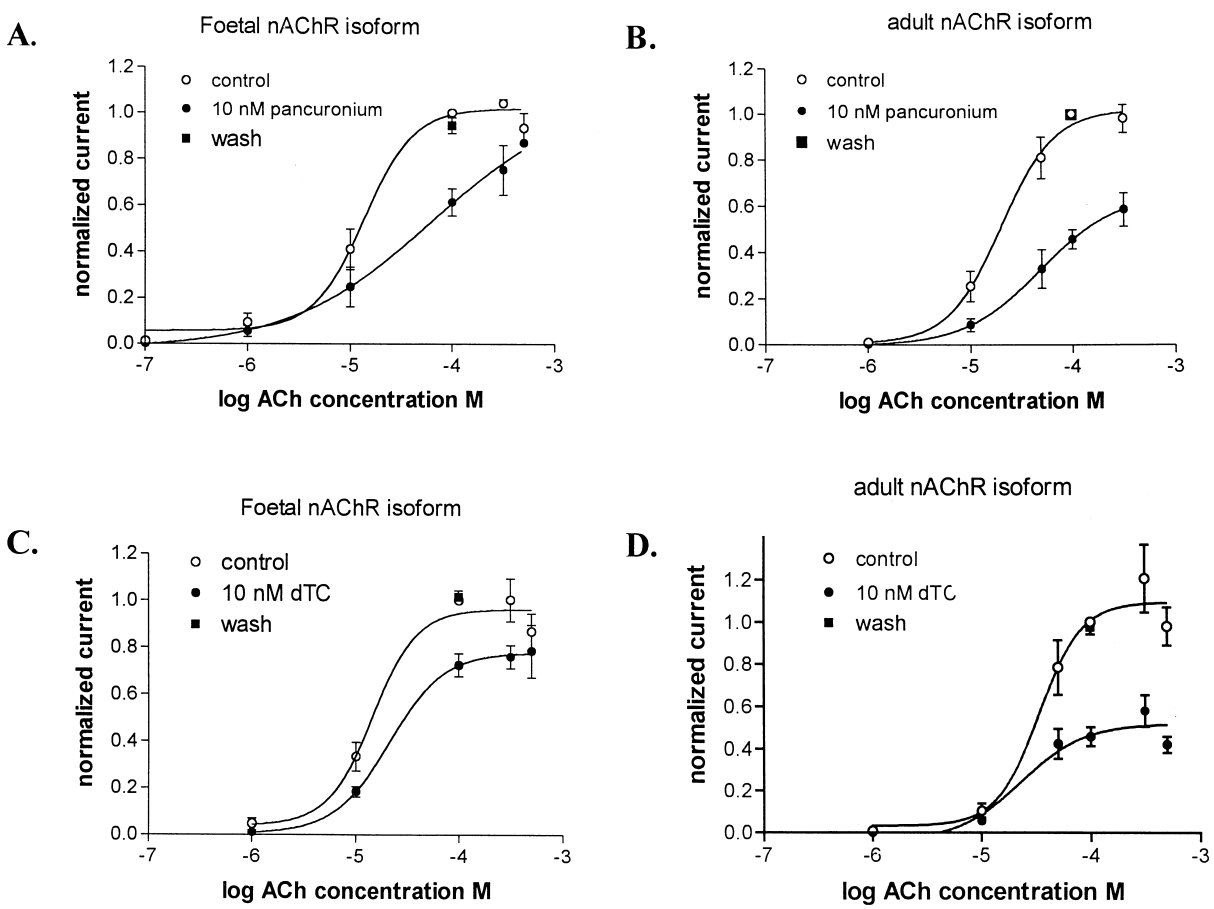

Fig. 3. The effect of d-tubocurarine (dTC) and pancuronium on acetylcholine (ACh) mediated currents at foetal and adult muscle-type nicotinic acetylcholine receptors (nAChRs) expressed in *Xenopus* oocytes. Oocytes are voltage clamped at −80 mV. Data were normalized to the current response for 100 μM acetylcholine. Data points are means ± S.E.M. (A) Effect of 10 nM pancuronium on foetal muscle nicotinic acetylcholine receptors, $n = 8$. (B) Effect of 10 nM pancuronium of adult muscle nicotinic acetylcholine receptors, $n = 4$–10. (C) Effect of 10 nM dTC on foetal muscle nicotinic acetylcholine receptors, $n = 7$. (D) Effect of 10 nM dTC on adult muscle nicotinic acetylcholine receptors, $n = 6$–21.

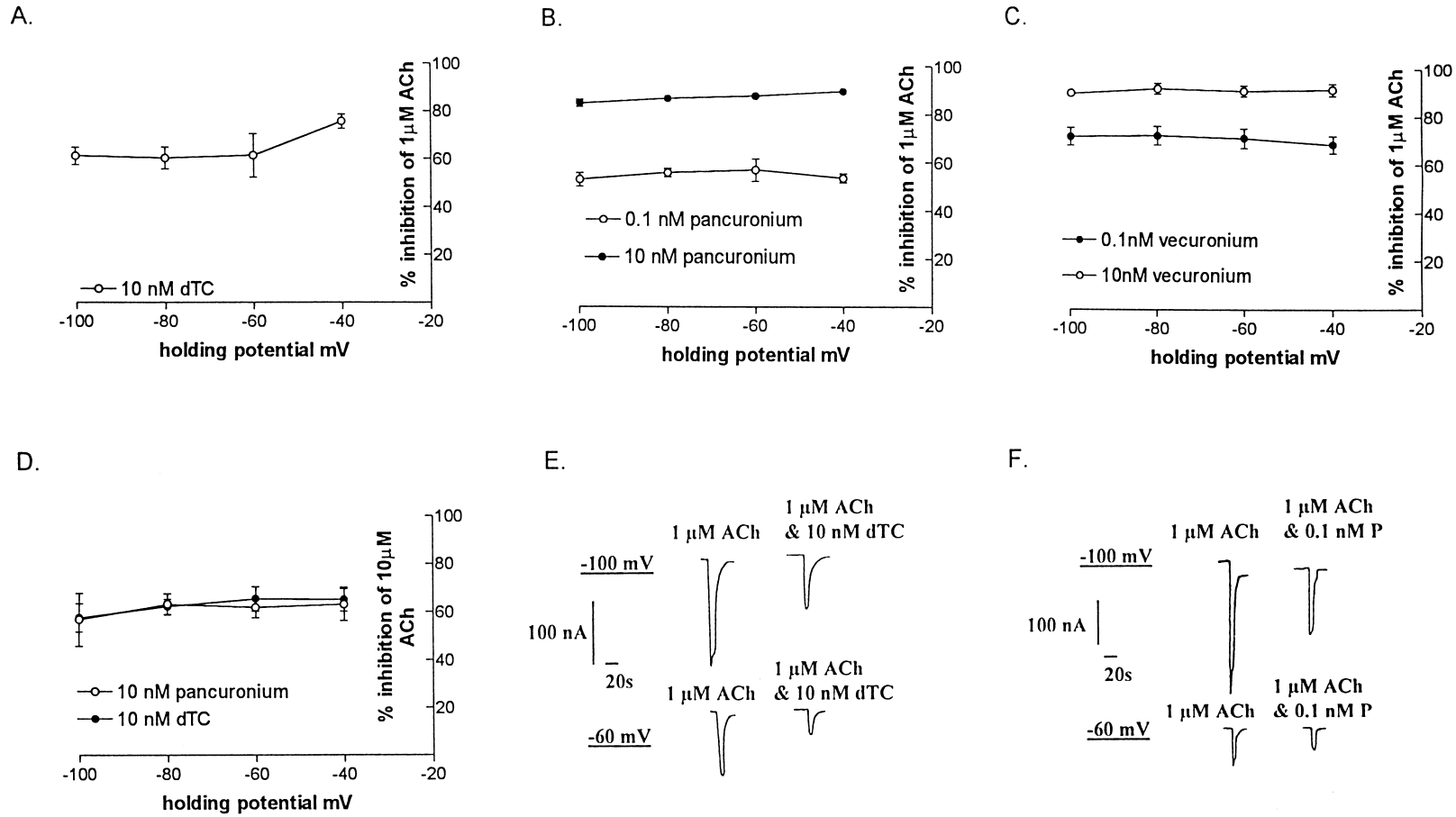

Fig. 4. Effect of the holding potential on the inhibition by pancuronium, d-tubocurarine (dTC) and vecuronium. Oocytes expressing foetal or adult muscle type nicotinic acetylcholine receptors (nAChRs) were voltage clamped at potentials ranging from −100 to −40 mV. The responses to acetylcholine (ACh; 1 μM foetal; 10 μM adult) were recorded in the presence and absence of dTC, pancuronium or vecuronium. (A) Effect of 10 nM dTC on foetal muscle nicotinic acetylcholine receptor, $n = 5$. (B) Effect of 0.1 nM ($n = 3$) and 10 nM ($n = 4$) pancuronium on foetal nicotinic acetylcholine receptors. (C) Effect of 0.1 nM ($n = 8$) and 10 nM ($n = 3$) vecuronium on foetal nicotinic acetylcholine receptors. (D) Effect of 10 nM dTC ($n = 5$) and 10 nM ($n = 5$) pancuronium on adult muscle nicotinic acetylcholine receptors. (E) Example traces of the block of the acetylcholine current by dTC at the holding potentials of −100 and −60 mV. (F) Example traces of the block of the acetylcholine current by pancuronium (P) at the holding potentials of −100 and −60 mV.

($n = 8$). Although the inhibition by pancuronium was apparently greater, this did not achieve statistical significance. Furthermore, at 500 μM acetylcholine there was no significant difference in the magnitude of the current response in the presence of antagonist (Fig. 3A,C). However, in the adult form of the muscle nicotinic acetylcholine receptor the inhibition of the response to 100 μM acetylcholine by 10 nM d-tubocurarine and 10 nM pancuronium was greater than that for the foetal receptor ($55 \pm 5\%$, $n = 12$, $P < 0.01$; and $60 \pm 4\%$, $n = 10$, $P < 0.001$, respectively) and both antagonists also caused a significant reduction in the response to 500 μM acetylcholine (from 1438 ± 323 to 1025 ± 306 nA, $n = 4$, for pancuronium, and from 1528 ± 697 to 673 ± 337 nA, $n = 6$ for d-tubocurarine; $P < 0.05$, paired Students' *t*-test; Fig. 3B,D). The inhibition produced by pancuronium and d-tubocurarine was reversible after a 20 min wash (Fig. 3).

In a further series of experiments, threshold concentrations of acetylcholine for foetal (1 μM) and adult (10 μM) were used to determine whether the antagonism by either d-tubocurarine or pancuronium was voltage-dependent. The effects of both pancuronium and d-tubocurarine were found to be independent of holding voltage over the range from −100 to −40 mV (Fig. 4A,B,D). For these experiments oocytes were stepped at 20 mV intervals between −100 and −40 mV and the response to acetylcholine was determined at each potential in the presence and absence of antagonist. The percentage reduction in the size of the acetylcholine-elicited response was determined at each holding potential. The percentage reduction was not significantly different for each antagonist at each holding potential ($P > 0.05$).

3.2. The effect of vecuronium, a structural analogue of pancuronium, on adult and foetal muscle nicotinic receptors

Vecuronium (1 nM) reduced the response to acetylcholine equieffectively for both the foetal and adult isoforms of the nicotinic receptor (Fig. 5A). The response to 100 μM acetylcholine was reduced by $71 \pm 6\%$ ($n = 4$) and $71 \pm 5\%$ ($n = 10$), respectively ($P > 0.05$). Vecuronium, as with pancuronium, blocked the response to acetylcholine in a reversible, voltage-independent manner (Fig. 4C).

3.3. The effect of pancuronium and d-tubocurarine on neuronal nicotinic acetylcholine receptors

Acetylcholine was applied to oocytes expressing the neuronal nicotinic acetylcholine receptor combination α4β2, voltage clamped at −80 mV. As with the muscle type receptors, acetylcholine produced concentration-dependent inward currents with an EC_{50} of 19.1 μM (2.5 to 144; $n = 6$) and a maximal current of 194 nA (135 to 252; $n = 6$; Fig. 6A). 10 nM d-tubocurarine inhibited the current elicited by 100 μM acetylcholine by $30 \pm 5\%$ ($n = 6$) compared to 10 nM pancuronium which was more potent and inhibited the response by $69 \pm 6\%$ ($n = 6$; $P < 0.05$ compared to d-tubocurarine; Fig. 6B). Therefore, at this neuronal nicotinic acetylcholine receptor, d-tubocurarine is significantly less potent than pancuronium.

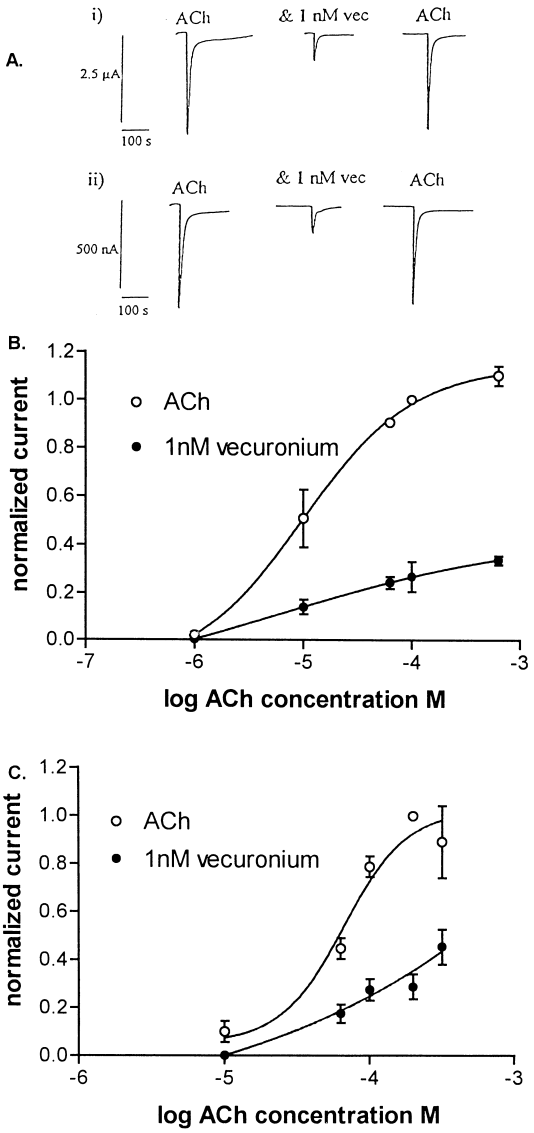

Fig. 5. The effect of vecuronium (vec) on acetylcholine (ACh) mediated currents at foetal and adult muscle-type nicotinic acetylcholine receptors expressed in *Xenopus* oocytes. (A) An example of the inhibition of the response to acetylcholine in (i) foetal and (ii) adult receptors by 1 nM vecuronium on oocytes voltage-clamped at −80 mV. 100 μM Acetylcholine was applied for 30 s. (B) The effect of 1 nM vecuronium on foetal muscle nicotinic acetylcholine receptors. Oocytes were voltage clamped at −80 mV. Data were normalized to the current response for 100 μM acetylcholine. Data points are means ± S.E.M., $n = 4$. (C) The effect of 1 nM vecuronium on adult muscle nicotinic acetylcholine receptors. Oocytes were voltage clamped at −80 mV. Data were normalized to the current response for 100 μM acetylcholine. Data points are means ± S.E.M., $n = 10$.

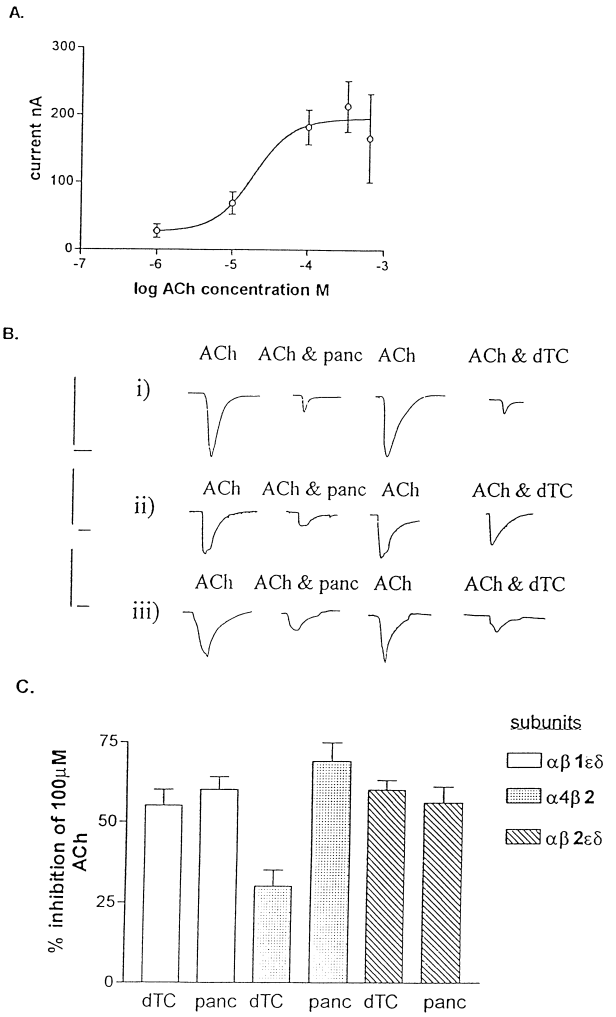

Fig. 6. The effect of d-tubocurarine (dTC) and pancuronium (panc) on neuronal α4β2 nicotinic acetylcholine receptors expressed in *Xenopus* oocytes. (A) Acetylcholine (ACh) elicited concentration-dependent inward currents in *Xenopus* oocytes expressing α4β2 nicotinic acetylcholine receptor subunits. Oocytes were voltage clamped at −80 mV. Data points are means ± S.E.M., $n = 7$. (B) Examples of recordings showing a comparison of the inhibition of the response to 100 μM acetylcholine for (i) αβ1εδ (ii) α4β2 and (iii) αβ2εδ nicotinic acetylcholine receptor by 10 nM pancuronium and 10 nM dTC. In each case acetylcholine was applied for 30 s and the antagonist was applied for 3 min prior to, and during, the acetylcholine application. (C) The percentage inhibition by 10 nM d-tubocurarine (dTC) and 10 nM pancuronium (panc) of the response to 100 μM acetylcholine was determined in oocytes expressing the nicotinic acetylcholine receptor subunit combinations αβ1εδ (dTC $n = 12$; panc $n = 10$), α4β2 (dTC $n = 6$; panc $n = 6$)and with the β subunit substitution, αβ2εδ (dTC $n = 6$; panc $n = 6$). Bars show mean ± S.E.M. There was no significant difference between the % inhibitions caused by dTC and pancuronium in αβ1εδ or αβ2εδ, however dTC caused significantly less inhibition than pancuronium ($P < 0.05$) for the α4β2 combination.

3.4. The effect of substituting the neuronal β2 subunit into the adult muscle nicotinic acetylcholine receptor on muscle relaxant pharmacology

The neuronal β2 subunit was substituted for the muscle β subunit in the adult muscle nicotinic acetylcholine receptor, giving a functional receptor of the subunit combination, αβ2εδ. 100 μM acetylcholine activated this receptor eliciting an average current of 343 ± 16 nA ($n = 6$). Both pancuronium and d-tubocurarine blocked the response elicited by 100 μM acetylcholine, with percentage inhibitions that were not significantly different from each other (pancuronium 56 ± 5%, $n = 6$; d-tubocurarine 60.0 ± 2.8%, $n = 6$; $P > 0.05$). These percentage inhibitions for the αβ2εδ were not significantly different from those observed in the adult muscle receptor subunit combination αβ1εδ (Fig. 6C; $P > 0.05$).

4. Discussion

The relative potency of muscle relaxants at subtypes of nicotinic acetylcholine receptors is most important from a clinical viewpoint. Here we have used cDNAs encoding for the receptor subunits and the *Xenopus* oocyte expression system to investigate the action of the muscle relaxants d-tubocurarine, pancuronium and vecuronium at nicotinic acetylcholine receptor subtypes. This is the first time these studies have been reported for vecuronium, which is now in widespread use as a short-acting muscle relaxant.

In the first instance we established the action of acetylcholine at these nicotinic acetylcholine receptor isoforms. The EC_{50} values were in close agreement to those obtained in previous studies (Labarca et al., 1995; Vibat et al., 1995). d-Tubocurarine and pancuronium blocked all three isoforms of the nicotinic acetylcholine receptor that we investigated, though there were some significant differences. For the foetal isoform, pancuronium and d-tubocurarine were equipotent. The antagonism was voltage-independent and the antagonists did not significantly decrease the response to the maximum concentration of acetylcholine. This leads us to suggest, in accordance with others (Neubig and Cohen, 1979; Filatov et al., 1993; Fletcher and Steinbach, 1996), that these neuromuscular blocking agents act in a competitive manner by binding to the acetylcholine-binding sites on nicotinic acetylcholine receptors. However, for the adult isoform of the muscle nicotinic acetylcholine receptor, both pancuronium and d-tubocurarine were more potent at blocking the response to acetylcholine and, although the block was voltage-independent, there was a significant reduction in the maximum response to acetylcholine. Previous studies characterising the interaction of muscle relaxants with the αβεδ subunit combination are restricted to radioligand binding assays on transfected quail fibroblasts (Fletcher and Steinbach, 1996) and have not clearly shown whether the interaction of the muscle relaxants with this isoform is competitive in nature. These observations indicate that the mechanism of interaction of d-tubocurarine and pancuronium with the adult muscle nicotinic acetylcholine receptor may be more complex than a simple competitive block.

While the mechanism of interaction for these antagonists is still equivocal, it is clear from the results presented here that the developmental switch of the nicotinic acetylcholine receptor from the γ to the ε subunit alters the antagonism of the nicotinic acetylcholine receptor for both pancuronium and d-tubocurarine with the ε subunit conferring greater sensitivity to d-tubocurarine and pancuronium than the δ subunit. A similar relative potency for these compounds has been observed for foetal and adult nicotinic acetylcholine receptors stably expressed in transfected quail fibroblasts (Fletcher and Steinbach, 1996). Using this approach, it has also been suggested that d-tubocurarine is less effective than pancuronium at blocking the foetal nicotinic acetylcholine receptors (Kopta and Steinbach, 1994; Fletcher and Steinbach, 1996). It was proposed that this was due to a partial agonist effect of d-tubocurarine on the foetal nicotinic acetylcholine receptor (Steinbach and Chen, 1995), a property which pancuronium does not exhibit (Fletcher and Steinbach, 1996). However, we did not observe any direct effect of d-tubocurarine on foetal nicotinic acetylcholine receptors and although the inhibition by pancuronium was apparently greater than that by d-tubocurarine, this was not statistically significant.

Vecuronium, a close structural analogue of pancuronium, differing only in that it has tertiary rather than a quaternary nitrogen, was more potent than either d-tubocurarine or pancuronium at both the foetal and adult nicotinic acetylcholine receptor subunit combinations. This observation indicates that the greater potency of vecuronium than pancuronium and d-tubocurarine in vivo (Savage et al., 1980; Fahey et al., 1981) may derive, at least in part, from its higher affinity for the muscle nicotinic acetylcholine receptor. In contrast to d-tubocurarine and pancuronium, the potency of vecuronium was not affected by swapping the δ subunit for an ε subunit and it blocked both isoforms equi-effectively. Thus, the tertiary nitrogen must enable a high affinity binding reaction with both the foetal and the adult nicotinic acetylcholine receptors.

The most abundant nicotinic acetylcholine receptor in the brain is composed of α4β2 subunits (Whiting and Lindstrom, 1987, 1988). Under normal physiological conditions, these receptors are not readily accessible to non-depolarising muscle relaxants as these drugs are highly charged and do not cross the blood brain barrier (Cardone et al., 1994). However, critically ill patients can be susceptible to pathophysiological disruptions of the blood–brain barrier, hence allowing neuromuscular blocking agents into the central nervous system. For example, d-tubocurarine has been found in cerebrospinal fluid after a single intravenous dose (Matteo et al., 1977), and vecuronium has been reported in the cerebrospinal fluid of patients with sepsis who had undergone prolonged vecuronium administration (Segredo et al., 1990). Under these conditions, the potency of muscle relaxants at neuronal subtypes of nicotinic receptors becomes clinically important. We have shown that at the α4β2 nicotinic acetylcholine receptor expressed in *Xenopus* oocytes, d-tubocurarine was significantly less effective at blocking the acetylcholine-elicited response than pancuronium. This is in contrast to the adult muscle nicotinic acetylcholine receptor, where both antagonists were shown to be equipotent. As outlined in the introduction, the major determinants of binding at muscle nicotinic acetylcholine receptors are the α, γ (or ε) and δ subunits although it has also been shown that substitution of the neuronal β2 subunit into the muscle nicotinic acetylcholine receptor can confer a neuronal-like pharmacology on the receptor (Wheeler et al., 1993, 1994). To investigate whether the neuronal β2 subunit could alter the pharmacology of the muscle nicotinic acetylcholine receptor with respect to d-tubocurarine and pancuronium, we expressed the neuronal β2 subunit in place of the usual muscle β1. The resulting hybrid receptor had the same sensitivity to d-tubocurarine and pancuronium as the αβ1εδ subunit combination, indicating that the β1 and β2 subunits are pharmacologically indistinguishable in these terms. This further supports the idea that the actions of these neuromuscular blocking compounds is determined by the acetylcholine binding sites which are at the α–γ (or ε) and α–δ interfaces (Caldwell et al., 1995) and indicates that the α4 subunit is the critical determinant differentiating between d-tubocurarine and pancuronium in the neuronal nicotinic acetylcholine receptor isoform.

Acknowledgements

We are grateful to Organon Teknika for financial support, to Professor S. Heinemann for providing cDNAs for the foetal nicotinic acetylcholine receptor subunits, to Dr. P. Gardner for the cDNA for the ε subunit, and to Professor J. Lindstrom for the cDNAs for the neuronal nicotinic acetylcholine receptor subunits. We are also grateful to Dawn McClean for technical help.

References

Baird, M., Reid, M., 1967. The neuromuscular blocking properties of a new steroid compound, pancuronium bromide. Br. J. Anaesthesia 39, 775–780.

Blount, P., Merlie, J.P., 1989. Molecular basis of the two non-equivalent ligand binding sites of the muscle nicotinic acetylcholine receptor. Neuron 3, 349–357.

Boulter, J., Evans, K., Goldman, D., Martin, G., Treco, D., Heinemann, S., Patrick, J., 1986. Isolation of a cDNA clone coding for a possible neuronal nicotinic acetylcholine receptor alpha subunit. Nature 319, 369–374.

Buckett, W.R., 1968a. The pharmacology of pancuronium bromide: a new non-depolarising blocking agent. Ir. J. Med. Sci. 7th Series 1, 565–568.

Buckett, W.R., 1968b. The pharmacology of pancuronium bromide (ORG.NA97), a new potent steroidal neuromuscular blocking agent. Br. J. Pharmacol. Chemother. 32, 671–682.

Caldwell, W.S., Schmitt, J.D., Benhert, M., Robinson, J.H., Lipiello, P.M., 1995. A combinatorial model of the nicotinic acetylcholine receptor pharmacophore. Soc. Neurosci. Abstr. 21, 11–36.

Cardone, C., Szenohradsky, J., Yosy, S., Bickler, P.E., 1994. Activation of brain acetylcholine receptors by neuromuscular blocking drugs. Anesthesiology 80, 1155–1161.

Deneris, E.S., Connolly, J., Boulter, J., Wada, E., Wada, K., Swanson, L.W., Patrick, J., Heinemann, S., 1988. Primary structure and expression of beta-2—a novel subunit of neuronal nicotinic acetylcholine-receptors. Neuron 1, 45–54.

Deneris, E.S., Connolly, J., Rogers, S.W., Duvoisin, R., 1991. Pharmacological and functional diversity of neuronal nicotinic acetylcholine receptors. Trends in Pharmacological Science 12, 34–40.

Elgoyhen, A.B., Johnson, D.S., Boulter, J., Vetter, D.E., Heinemann, S., 1994. ALPHA9: an acetylcholine receptor with novel pharmacological properties expressed in rat cochlear hair cells. Cell 79, 705–715.

Fahey, M.R., Morris, R.B., Mille, R.D., Connelly, R., Gencarell, P., 1981. Clinical pharmacology of OrgNC45 (Norcuron). Anesthesiology 55, 6–11.

Filatov, G.N., Aylwin, M.L., White, M.M., 1993. Selective enhancement of the interaction of curare with the nicotinic acetylcholine receptor. Mol. Pharmacol. 44, 237–241.

Fletcher, G.H., Steinbach, J.H., 1996. Ability of non-depolarizing neuromuscular blocking drugs to act as partial agonists at foetal and adult mouse muscle nicotinic receptors. Mol. Pharmacol. 49, 938–947.

Gerzanich, V., Anand, R., Lindstrom, J., 1993. Homomers of alpha8 and alpha7 subunits of nicotinic receptors exhibit similar channel but contrasting binding site properties. Mol. Pharmacol. 45, 212–220.

Goldman, D., Deneris, E., Luyten, W., Kochhar, A., Patrick, J., Heinemann, S., 1987. Members of a nicotinic acetylcholine receptor gene family are expressed in different regions of the mammalian CNS. Cell 48, 965–973.

Harvey, S.C., Luetje, C.W., 1996. Determinants of competitive antagonist sensitivity on neuronal nicotinic receptor-beta subunits. J. Neurosci. 16, 3798–3806.

Harvey, S.C., Maddox, F.N., Luetje, C.W., 1996. Multiple determinants of dihydro-beta-erythroidine sensitivity on rat neuronal nicotinic receptor-alpha subunits. J. Neurochem. 67, 1953–1959.

Kao, P.N., Dwork, A.J., Kaldany, R.R.J., Silver, M.L., Wideman, J., Stein, S., Karlin, A., 1984. Identification of the alpha-subunit half-cysteine specifically labelled by an affinity reagent for the acetycholine receptor binding site. J. Biol. Chem. 259, 11662–11665.

Kopta, C., Steinbach, J.-H., 1994. Comparison of mammalian adult and fetal nicotinic acetylcholine receptors stably expressed in fibroblasts. J. Neurosci. 14, 3922–3933.

Kurosaki, T., Fukuda, K., Kanno, T., Mishina, M., Numa, S.S., 1987. Functional properties of nicotinic acetylcholine receptor subunits expressed in various combinations. FEBS Lett. 214, 253–258.

Labarca, C., Nowak, M.W., Zhang, H., Tang, L., Deshpande, P., Lester, H.A., 1995. Channel gating governed symmetrically by conserved leucine residues in the M2 domain of nicotinic receptors. Nature 376, 514–516.

Luetje, C.W., Patrick, J., 1991. Both α- and β-subunits contribute to the agonist sensitivity of neuronal nicotinic acetylcholine receptors. J. Neurosci. 11, 837–845.

Luetje, C.W., Wada, K., Roger, S., Abramson, S.N., Tsuji, K., Heinemann, S., Patrick, J., 1990. Neurotoxins distinguish between different neuronal nicotinic acetylcholine receptor subunit combinations. J. Neurochem. 55, 632–640.

Matteo, R.S., Pua, E.K., Khambatta, H.J., Spector, S., 1977. Cerebrospinal fluid levels of d-tubocurarine in man. Anesthesiology 46, 396–399.

Mauron, A., Nef, P., Stadler, R., Alliod, C., Ballivet, M., 1985. Structure, linkage and sequence of the two genes encoding the delta-subunits and gamma-subunits of the nicotinic acetylcholine receptor. Proc. Natl. Acad. Sci. USA 81, 806–816.

Mishina, M., Takai, T., Imoto, K., Noda, M., Takahashi, T., Numa, S., Methfessel, C., Sakmann, B., 1986. Molecular distinctions between fetal and adult forms of muscle acetylcholine receptor. Nature 321, 406–411.

Neubig, R., Cohen, J.B., 1979. Equilibrium binding of ^{3}H-tubocurarine and ^{3}H-acetylcholine by *Torpedo* postsynaptic membrane: Stoichiometry and ligand interaction. Biochemistry 18, 5464–5475.

Neumann, D., Gershoni, J.M., Fridkin, M., Fuchs, S., 1985. Antibodies to synthetic peptides as probes for the binding site on the α-subunit of the acetylcholine receptor. Proc. Natl. Acad. Sci. USA 82, 3490–3493.

Neumann, D., Barchan, D., Safran, A., Gershoni, J.M., Fuchs, S., 1986. Mapping of the α-bungarotoxin binding site within the α-subunit of the acetylcholine receptor. Proc. Natl. Acad. Sci. USA 83, 3008–3011.

Noda, M., Takahashi, H., Tanabe, T., Moyosato, M., Furutani, Y., Hirose, T., Asai, M., Inayama, S., Miyata, T., Numa, S., 1982. Primary structure of α-subunit precursor of *Torpedo californica* acetylcholine receptor deduced from cDNA sequence. Nature 299, 793–797.

O'Leary, M.E., Filatov, G.N., White, M.M., 1994. Characterization of the D-tubocurarine binding site of Torpedo acetylcholine receptor. Am. J. Physiol. 266, 648–653.

Papke, R.L., Heinemann, S., 1993. Partial agonist properties of cytisine on neuronal nicotinic receptors containing the beta-2 subunit. Mol. Pharmacol. 45, 142–149.

Pederson, S.E., Cohen, J.B., 1990. D-Tubocurarine binding sites are located at alpha–gamma and alpha–delta subunit interfaces of the nicotinic acetylcholine receptor. Proc. Natl. Acad. Sci. USA 87, 2785–2789.

Reynolds, J.A., Karlin, A., 1978. Molecular weight in detergent solution of the acetylcholine receptor for *T.californica*. Biochemistry 17, 2035–2038.

Savage, D.S., Sleigh, T., Carlyle, J., 1980. The emergence of ORG NC45 from the pancuronium series. Br. J. Anaesth. 52, 3S–9S.

Schuetze, S.M., Role, L.W., 1987. Developmental regulation of nicotinic acetylcholine receptors. Annu. Rev. Neurosci. 10, 403–457.

Segredo, V., Matthay, M.A., Sharma, M.L., Gruenke, L.D., Caldwell, J.E., Miller, R.D., 1990. Prolonged neuromuscular blockade after long-term administration of vecuronium in two critically ill patients. Anesthesiology 72, 566–570.

Sine, S.M., 1993. Molecular dissection of subunit interfaces in the acetylcholine receptor—identification of residues that determine curare selectivity. Proc. Natl. Acad. Sci. USA 90, 9436–9440.

Sine, S.M., Claudio, T., 1991. γ-subunit and δ-subunit regulate the affinity and the cooperativity of ligand binding to the acetylcholine receptor. J. Biol. Chem. 266, 19369–19377.

Sine, S.M., Claudio, T., Sigworth, F.J., 1990. Activation of Torpedo acetylcholine receptors expressed in mouse fibroblasts—single channel current kinetics reveal distinct agonist binding affinities. J. Gen. Physiol. 96, 395–437.

Sine, S.M., Quiram, P., Paparikalou, F., Kreinkamp, H.J., Taylor, P., 1994. Conserved tyrosines in the α-subunit of the nicotinic acetylcholine receptor stabilise quaternary ammonium groups of agonists and curariform antagonists. J. Biol. Chem. 269, 8808–8816.

Steinbach, J.H., Chen, Q., 1995. Antagonist and partial agonist actions of d-tubocurarine at mammalian muscle nicotinic acetylcholine receptors. J. Neurosci. 15, 230–240.

Swanson, R., Folander, K., 1992. In vitro synthesis of RNA for expression of ion channels in *Xenopus* oocytes. Methods in Enzymology 207, 310–319.

Tomaselli, G.F., McLaughlin, J.T., Jurman, M.E., Hawrot, E., Yellen, G., 1991. Mutations affecting agonist sensitivity of the nicotinic acetylcholine receptor. Biophys. J. 60, 721–727.

Vibat, C.R.T., Lasalde, J.A., McNamee, M.G., Ochoa, E.L.M., 1995. Acetylcholine-receptor subunit combinations expressed in *Xenopus-laevis* oocytes. Cell. Mol. Neurobiol. 15, 411–425.

Weber, M., Changeux, J-P., 1974. Binding of *Naja nigricollis* ^{3}H-alpha

toxin to membrane fragments from *Electrophorus* and *Torpedo* electric organs: 1. Binding of the tritiated α- neurotoxin in the absence of effector. Mol. Pharmacol. 10, 1–4.

Wheeler, S.V., Chad, J.E., Foreman, R.C., 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. FEBS Lett. 332, 139–142.

Wheeler, S.V., Jane, S.D., Cross, K.M.L., Chad, J.E., Foreman, R.C., 1994. Membrane clustering and α-bungarotoxin binding by the nicotinic acetylcholine receptor—role of the β-subunit. J. Neurochem. 63, 1891–1899.

Whiting, P., Lindstrom, J., 1987. Purification and characterisation of a nicotinic acetylcholine receptor from rat brain. Proc. Natl. Acad. Sci. USA 84, 595–599.

Whiting, P., Lindstrom, J., 1988. Characterisation of bovine and human neuronal nicotinic receptors using monoclonal antibodies. J. Neurosci. 6, 3061–3069.