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Pharmacology of the nicotinic acetylcholine receptor from fetal rat muscle expressed in *Xenopus* oocytes

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Abstract

The fetal rat muscle nicotinic acetylcholine receptor was expressed in *Xenopus* oocytes. Using the voltage-clamp technique, the response to a range of agonists was measured, listed in order of (decreasing) activity efficacy: anatoxin ≥ epibatidine > acetylcholine > DMPP (1,1-dimethyl-4-phenylpiperazinium) ≫ cytisine > pyrantel > nicotine > coniine > tubocurare > lobeline. The agonist responses were compared with the steric and electrostatic properties of the molecules, using molecular modelling. Single-channel currents were measured in outside-out patches for acetylcholine, nicotine, cytisine, anatoxin and epibatidine. The conductance of the single channels was independent of the type of agonist. The mean open times were characteristic of the agonist applied. Tubocurare, better known for its antagonist properties, was also a partial agonist. Single-channel currents were also observed for tubocurare, and for methyllycaconitine in patches with a very high density of the muscle nicotinic acetylcholine receptor, and these were blocked by α-bungarotoxin. The agonist properties of physostigmine, galanthamine and their methyl derivatives were also investigated. The conductance of the channels observed in outside-out patches was similar to that obtained for the classical agonists. The single-channel currents observed for physostigmine, galanthamine and their methyl derivatives were blocked by α-bungarotoxin, methyllycaconitine and mecamylamine, in contrast to previously reported studies on neuronal and adult muscle nicotinic acetylcholine receptors.

Keywords: Nicotinic acetylcholine receptor; Muscle, fetal, rat; Patch-clamp; Voltage-clamp; Molecular modelling; Nicotinic agonist; Non-competitive agonist

1. Introduction

The functional and pharmacological characterisation of nicotinic acetylcholine receptors is an important goal, especially as these receptors may be suitable targets for drug development. One modern approach towards this goal is to develop a three-dimensional molecular model of substances that interact with the receptor. For this purpose, it is necessary to characterise the functional response of the receptor to a variety of different ligands, with particular emphasis on ligands that have a rigid conformation and therefore provide structural information.

We have chosen the nicotinic acetylcholine receptor from fetal rat muscle as a model receptor. This can be expressed in *Xenopus* oocytes (Witzemann et al., 1990), which are convenient for the functional investigation of the nicotinic acetylcholine receptor using voltage-clamp and patch-clamp techniques (Methfessel et al., 1986). Compared to other nicotinic receptors, this subtype is particularly easy to study in oocytes because it gives high levels of expression and shows little desensitisation during prolonged application of moderate (i.e. sub-μM) agonist concentrations.

In addition to a set of classical nicotinic agonists, we have also investigated two compounds that were more recently found to be potent nicotinic acetylcholine receptor agonists: anatoxin (Spivak et al., 1980; Swanson et al., 1986) and epibatidine (Badio and Daly, 1994). Further, single-channel currents were observed for physostigmine, galanthamine and their methyl derivatives in outside-out patches from oocytes expressing the rat muscle nicotinic acetylcholine receptor. These ligands have been of recent interest due to their agonist activity at a second binding site on neuronal nicotinic acetylcholine receptors, independent of the acetylcholine binding site (Pereira et al., 1993a,b,1994; Albuquerque et al., 1991).

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2. Materials and methods

Acetylcholine chloride, (−)-nicotine, coniine, cytisine, L-lobeline chloride, gentamicin, α-bungarotoxin, *d*-tubocurare chloride, mecamylamine and physostigmine hemisulphate were obtained from Sigma (St. Louis, MO, USA). Methylphysostigmine iodide and methylgalanthamine iodide were synthesised by Dr. Lensky (Zentrale Forschung, Bayer, Leverkusen, Germany) using methyl iodide as the methylating agent. The correct structure of the purified reaction products was confirmed by MS-FAB. DMPP iodide (1,1-dimethyl-4-phenylpiperazinium iodide) was purchased from Aldrich (UK). Pyrantel pamoate was a gift from Dr. Londershausen (Bayer, Monheim, Germany). (±)-Epibatidine dihydrochloride, (+)-anatoxin A and methyllycaconitine citrate were obtained from RBI (Natick, MA, USA). Galanthamine hydrobromide was purchased from Latoxan (Rosans, France). All agonists and antagonists, with the exception of lobeline, coniine, pyrantel and DMPP were stored as aqueous stock solutions, aliquoted and kept frozen until they were further diluted in normal frog Ringer solution on the day of use. Fresh solutions of lobeline, coniine and DMPP were made in normal frog Ringer solution on the day of measurement. A 10 mM solution of pyrantel in DMSO (dimethylsulphoxide) was made on the day of use, and this was further diluted with normal frog Ringer solution as required.

The cRNA coding for fetal rat muscle nicotinic acetylcholine receptor subunits was prepared as described previously (Witzemann et al., 1990) and mixed for injection in the ratio $\alpha_1 : \beta_1 : \gamma : \delta = 2 : 1 : 1 : 1$.

2.1. Expression of channels in oocytes

Xenopus laevis oocytes in stage V or VI were obtained and injected with cRNA as described previously (Methfessel et al., 1986). Oocytes were incubated for 2 h with collagenase (Sigma Type I, EC 3.4.24.3, 1 mg/ml in calcium-free Barth's medium) to dissociate the ovary and remove the follicle cell layer before injection. About 50 nl of cRNA solution containing 100 or 250 ng/μl RNA was injected into each oocyte. Oocytes were incubated for 3–14 days at 19°C in Barth's medium (in mM: NaCl 88, $NaHCO_3$ 2.4, KCl, 1.0, $Ca(NO_3)_2$ 0.33, $CaCl_2$ 0.41, $MgSO_4$ 0.82, Tris/HCl 5.0, pH 7.4) containing 50 μg/ml gentamicin. The culture medium was changed three times a week.

2.2. Whole-cell current recordings

Whole-cell current records were obtained with the standard two electrode voltage-clamp technique. Glass microelectrodes were pulled from aluminosilicate glass (Hilgenberg, Malsfeld, Germany) using a standard vertical puller (L/M-3P-A; List-Medical, Heidelberg, Germany). The pipette resistance was about 0.3–0.5 MΩ when filled with the solution containing 1.5 M potassium acetate and 100 mM potassium chloride. During measurements the recording chamber was perfused at approximately 10 ml/min with a normal frog Ringer solution of the following composition in mM: NaCl 115, KCl 2.5, $CaCl_2$ 1.8, Hepes 10, pH 7.2. Using electromagnetic valves controlled by a personal computer, the perfusion solution could be changed to normal frog Ringer solution containing a substance of interest. Whole-cell currents were measured with a Tec 01-02 cc amplifier (npi electronic, Tamm, Germany) at −80 mV, digitized on-line and stored on the computer for later analysis. In order to correct for variability between oocytes and/or run-down of response, the inward current due to 0.1 μM acetylcholine was used as a reference signal. The responses of substances of interest are thus given relative to a prior application of 0.1 μM acetylcholine. Hill coefficients were determined as the slope of the dose-response relationship in a double logarithmic representation.

2.3. Single-channel recording and analysis

For single-channel recording, the vitelline layer was removed from oocytes as described previously (Methfessel et al., 1986). The oocytes were incubated for a few minutes in the following solution which was hypertonic with respect to Barth's solution (in mM): K-aspartate 200, KCl 20, $MgCl_2$ 1, EGTA 10, Hepes 10, pH 7.4. The vitelline layer was then removed using forceps, and the oocyte was transferred to a culture dish containing normal frog Ringer solution. Patch pipettes were pulled from thin-walled borosilicate glass capillaries with external diameter 1.6 mm (Hilgenberg, Malsfeld, Germany). The pipettes were coated with silicone and the tips were fire-polished on a microforge. The pipette resistance was 7–8 MΩ when filled with the following internal solution (in mM): KF 80, KCl 20, EGTA 10, Hepes 10, pH 7.2, which was used as pipette solution for all outside-out patch recordings.

Substances of interest were dissolved in normal frog Ringer solution and applied to the outside-out patches using a double-barrelled glass theta tube, pulled and bent to an appropriate shape (Spitzer and Bridge, 1989). The solution flowing through one barrel was normal frog Ringer solution, while normal frog Ringer solution containing the substance or substances of interest was passed through the other barrel. The active barrel of the theta tube was connected to a rotary selector disc (Bormann, 1992) to allow rapid selection of the test solution from one of six reservoirs. Normal frog Ringer solution was always applied to the patch first, to check that no endogenous channels were present. Then, the test solution was applied from the second barrel. The chamber was continuously perfused with normal frog Ringer solution at 4–5 ml/min using a gravity flow arrangement.

All single-channel recordings were made at room temperature (21–23°C) using a LM-EPC7 patch-clamp ampli-

fier (List Electronic, Heidelberg, Germany). Data were stored on digital audio tape (DTR-1204 digital tape recorder, Biologic, Claix, France) for later analysis. The signals were filtered at 2.5 kHz (Bessel), digitised at 10 kHz and analysed on a personal computer with the pClamp (Version 6.0) software package (Axon Instruments, Foster City, CA). Open-channel events were considered terminated when the amplitude decreased to below 50% of the mean amplitude. Mean single-channel amplitudes were obtained by fitting a Gaussian function to the amplitude distribution. Time constants ($\tau 1$ and $\tau 2$) for the open-channel state were determined by maximum likelihood fitting of one or two exponential distributions to binned histograms (bin width 0.1 ms), with no correction for missed events. In order to obtain an estimate of the probability of channel opening P_0, the following approximation was used (Colquhoun and Sakmann, 1985):

$$P_0 = \frac{n}{t} \times \tau = f \times \tau$$

where n is the number of channel openings, t the total time of recording, f is frequency of channel openings and τ is mean open time of the channels; f is the total number of channel events divided by the time of recording, with simultaneous openings counted as two events.

2.4. Molecular modelling

Selected nicotinic acetylcholine receptor agonists were studied using a molecular modelling approach.

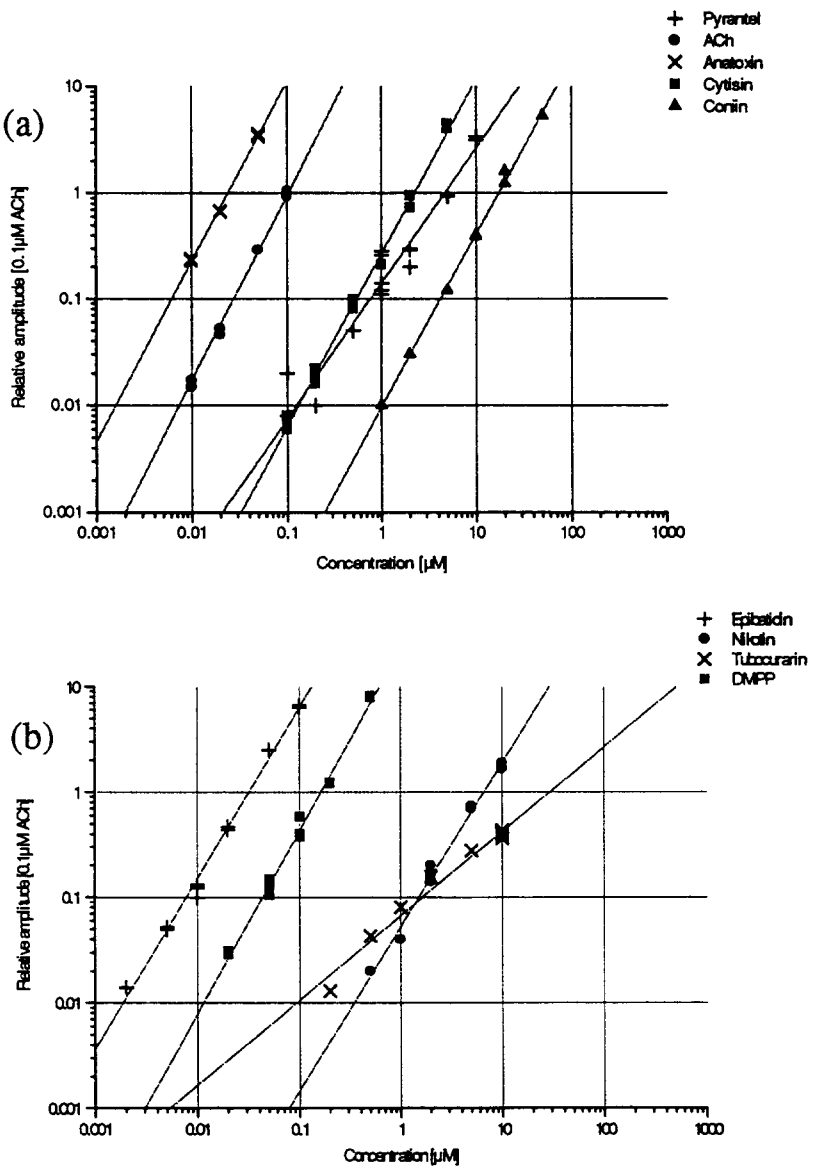

Fig. 1. Dose-response plots for different agonists measured by voltage-clamp on whole oocytes (a) coniine ▲, pyrantel +, cytisine ■, anatoxin ×, acetylcholine ● (b) nicotine ●, tubocurare ×, DMPP ■, epibatidine +. The inward current response is given relative to that for 0.1 µM acetylcholine, to compensate for variation between oocytes, and run-down during the course of an experiment.

For acetylcholine, anatoxin, cytisine, DMPP, nicotine and lobeline, crystal structures extracted from the Cambridge Structural Database (Allen et al., 1991) were used as a reference conformation and these were subjected to an optimization procedure using the semi-empirical program MOPAC 6.0 (J.J.P. Stewart, QCPE 455,93). Structural models of epibatidine, pyrantel and coniine were constructed with the SYBYL molecular modelling software (Tripos Association, St. Louis, MO, USA, 1995) program package and were refined in a similar manner.

A crucial step for all active analogue modelling is the alignment of the molecules under study. Superposition was performed based on the cationic centre, the 'bridge' atom next to the nitrogen and the corresponding lone-pair vector of the putative hydrogen acceptor group. One of the carbonyl oxygen lone pairs served as anchor point for acetylcholine, anatoxin, cytisine and lobeline whereas for epibatidine and nicotine, the pyridyl nitrogen took this role. DMPP and pyrantel were overlayed relative to nicotine using the centre of the phenyl or thiophene, respectively, as an alternative anchor point.

3. Results

3.1. Activity of different agonists

3.1.1. Selected nicotinic acetylcholine receptor agonists

The responses of the rat fetal muscle nicotinic acetylcholine receptor to selected agonists were investigated using whole-cell voltage-clamp on oocytes expressing the receptor. The relative inward current response to various concentrations of each substance was measured against 0.1 μM acetylcholine as described under Materials and methods. These data were used to produce dose-response curves (Fig. 1), and to calculate Hill coefficients, which are shown in Table 1, together with the concentration, given as $-\log_{10}$, required to produce a response equivalent to 0.1 μM acetylcholine. The classical agonists show a Hill coefficient close to 2, indicating that binding of two agonist molecules at each receptor is required to fully activate the channel.

Table 1
Whole-cell current responses to various agonists measured with the voltage-clamp method

Substance	$-\log_{10}$ (concentration) to give response equivalent to 0.1 μM acetylcholine	Hill coefficient
Anatoxin	7.6	1.70
Epibatidine	7.5	1.74
Acetylcholine	7.0	1.74
DMPP	6.8	1.73
Cytisine	5.7	1.63
Pyrantel pamoate	5.3	1.28
Nicotine	5.2	1.55
Coniine	4.8	1.63
Tubocurare	4.5	–
Lobeline	< 4	–

The results are given as the concentration required for a response equivalent to 0.1 μM acetylcholine (1st column), or as the Hill coefficient (2nd column).

Table 2
Pharmacophoroeic distances and steric volumes (relative to anatoxin ⊕ epibatidine) for selected nicotinic acetylcholine receptor agonists

Substance	Pharmacologically relevant distances (Å)					Volume relative to anatoxin/epibatidine	
						Missing	Extra
Anatoxin	C=O⟨ ⋮ N⁺	3.0/4.6	Phenyl^{centre} - - - N⁺	4.2		–	–
Epibatidine	N⟶ - - N⁺	5.2				–	–
Acetylcholine	C=O⟨ ⋮ N⁺	4.4/6.0				114	39
DMPP			Phenyl^{centre} - - - N⁺	5.7		95	67
Cytisine	C=O⟨ ⋮ N⁺	4.5/5.7	Phenyl^{centre} - - - N⁺	3.9		92	40
Pyrantel	S⟨ ⋮ N⁺	6.2/6.5	Thiophe^{centre} - - - N⁺	5.9		98	67
Nicotine	N⟶ - - N⁺	5.2	Pyridyl^{centre} - - - N⁺	3.7		109	44
Coniine			Aliphat. terminus N⁺	5.0		92	17
Lobeline	C=O⟨ ⋮ N⁺	2.3/3.7	Phenyl^{centre} - - - N⁺	5.2		90	135

Crystal structures for acetylcholine, anatoxin, cytisine, DMPP, nicotine and lobeline were obtained from the Cambridge Structural Database and were subjected to an optimisation procedure using the semi-empirical molecular orbital calculation program MOPAC 6.0. Structural models for epibatidine, pyrantel and coniine were constructed using the SYBYL program and were refined in a similar manner.

As a step towards elucidation of the structure-activity relationship of these agonists, a molecular modelling study was performed using an active analogue approach. The steric properties of the agonists were analyzed based on the alignment described under Materials and methods. The steric volumes of anatoxin and epibatidine, which are both rigid and very active, were combined and subtracted from the corresponding volume of each of the other agonists. The relevant pharmacophoric distances and the steric volumes in comparison to anatoxin/epibatidine are summarized in Table 2.

Of all the agonists in our selection, anatoxin has the highest agonist activity. We therefore superposed individual agonists upon the anatoxin molecule to look for possible structural differences that may influence relative agonist strength. Fig. 2a shows the superposition of anatoxin and epibatidine. Only minor differences are seen in the steric bulk close to the corresponding hydrogen bonding acceptor groups. DMPP, which is about an order of magnitude less active than anatoxin, is quite similar in shape to

both anatoxin and epibatidine (Fig. 2b) but lacks the important hydrogen bond acceptor group. Superposition of cytisine on anatoxin (Fig. 2c) demonstrates that the two six-membered ring moieties of cytisine require additional steric volume which may cause the drastic drop in agonist activity. Pyrantel is a slightly weaker agonist: the pharmacophoric groups (thiophene moiety, positively charge nitrogen) are separated by more than 6 Å compared to 5 Å for the corresponding distance in anatoxin or epibatidine (Table 2). Nicotine has relatively low activity although its pyridyl group can act as a hydrogen bonding acceptor group. Although the spatial arrangement of the functional groups is quite similar to anatoxin and epibatidine, much of the steric volume present in these agonists is absent in nicotine due to the relatively small pyrrolidine moiety (Table 2). The rather low activity of coniine can easily be explained by the lack of a vital hydrogen bonding acceptor group. Lobeline's extremely low dose-response activity results from the large excess volume introduced by the hydroxybenzyl moiety. Additional bulk also appears close to the hydrogen bonding acceptor function (Table 2).

3.1.2. Tubocurare

Tubocurare is usually classed as an antagonist of the nicotinic acetylcholine receptor. However, tubocurare alone (0.2–10 μM) also produced an inward current response (Fig. 1), which was not observed for non-injected control oocytes (3 oocytes), or in cRNA injected oocytes that were incubated for 15 min in 100 nM α-bungarotoxin. The maximal whole-cell currents obtained with tubocurare remained well below those activated by other agonists and the slope of the plot of relative response against tubocurare concentration was much less steep than for the classical agonists. This indicates that tubocurare is a partial agonist for the fetal rat muscle nicotinic acetylcholine receptor, confirming previous reports (Ziskind and Dennis, 1978; Morris et al., 1982; Trautmann, 1982; Jackson et al., 1982). No measurable whole-cell response was observed on application of 0.5–100 μM methyllycaconitine (2 oocytes).

3.1.3. Non-competitive agonists

Following reports that several classical acetylcholinesterase inhibitors can act as agonists at a second binding site on nicotinic acetylcholine receptors (Albuquerque et al., 1991; Pereira et al., 1993a,b,1994), we have studied the response of whole oocytes to physostigmine and methylgalanthamine using voltage-clamp. No response was observed to physostigmine from 0.1 μM to 1 mM (6 oocytes), or 100 μM methylgalanthamine (5 oocytes) even for oocytes that responded to 0.1 μM acetylcholine with more than 1 μA of current. Only the non-competitive antagonist activity expected of physostigmine-related substances (Shaw et al., 1985; Albuquerque et al., 1988) was observed. Thus, the response to 0.1 μM acetylcholine was reduced by about 50% in the presence of 100 μM methylgalanthamine.

3.2. Single-channel recordings

3.2.1. Response to different nicotinic agonists

Single-channel responses of the fetal rat muscle nicotinic acetylcholine receptor to selected agonists were measured in outside-out patches from oocytes. Fig. 3 shows typical single-channel recordings observed for acetylcholine, nicotine, cytisine, anatoxin and epibatidine.

No single-channel currents were observed on applica-

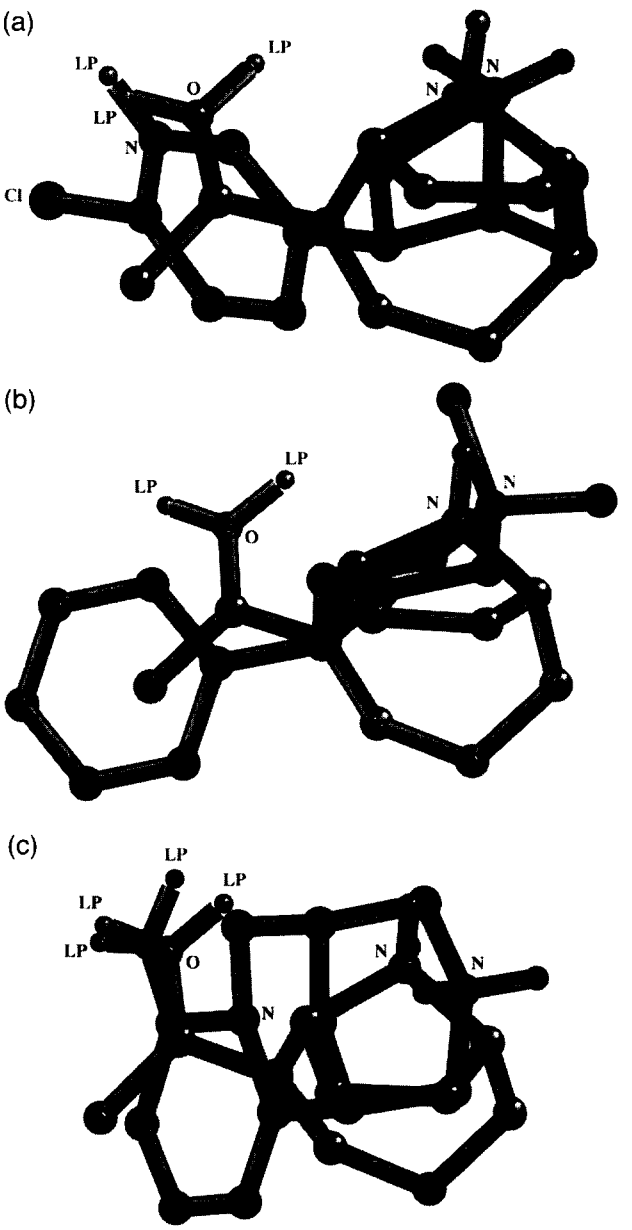

Fig. 2. Superposition of anatoxin (light) with (dark) (a) epibatidine (b) DMPP (c) cytisine. For anatoxin and cytisine, the anchor point was one of the carbonyl lone pairs (LP) whereas for epibatidine, the anchor point was the pyridyl nitrogen. DMPP was overlayed using the centre of the phenyl moiety as an anchor point.

tion of these substances to outside-out patches from oocytes that were previously incubated in 3 μM α-bungarotoxin for 1 h, or from non-injected control oocytes. The responses were not altered in the presence of 1 μM atropine.

3.2.1.1. Current amplitude analysis. Only single-channel events with durations longer than 0.5 ms were used for the amplitude analysis. Single-channel conductances were evaluated from the slopes of *i-V* plots of amplitude against membrane potential, as determined by a linear fit in the region of negative membrane potential. The single-channel conductances were practically the same for all agonists tested, in accordance with the assumption that all these agonists act on the same nicotinic acetylcholine receptor (in pS: acetylcholine: 36.8 ± 1.6; nicotine: 36.5 ± 0.9; cytisine: 36.4 ± 0.8; epibatidine: 37.9 ± 2.3; anatoxin: 36.6 ± 1.8). The mean conductance value was 36.8 ± 0.5 pS. All single-channel currents measured in these patches belonged to this conductance class; no evidence for other conductance levels or substates of the channel was seen.

3.2.1.2. Open-channel lifetimes. The mean open time of the single channels was determined by fitting the open-time histogram with two exponentials, giving two time constants $\tau 1$ and $\tau 2$ (see Table 3).

The longer time constant $\tau 2$ was clearly dependent on membrane potential. A strong dependence of $\tau 2$ on potential was observed for all agonists tested, with the exception of cytisine, for which this dependence was less pronounced (Fig. 4).

The time constants at -110 mV are given in Table 3. $\tau 2$ is longest for acetylcholine, followed by anatoxin and epibatidine albeit at a 10-fold lower concentration (it was not possible to use a higher concentration of these agonists for the open-time determination due to the high incidence of double and triple openings). The fraction of events fitted to the longer time constant is higher for anatoxin than for epibatidine. Nicotine and cytisine produced much shorter $\tau 2$ values although the concentration required was 10-fold higher. The fraction of events fitted to the longer time constant is much higher for cytisine than for nicotine.

Whereas the longer time constants are characteristic of the agonist applied, little variation was observed in the short time constant for different agonists. However, since the data were filtered at 2.5 kHz before analysis, these short lifetimes were not sufficiently resolved and no conclusions can be drawn about this finding.

3.2.2. Tubocurare

Consistent with the partial agonist activity of tubocurare in whole-cell recording, single-channel currents were also

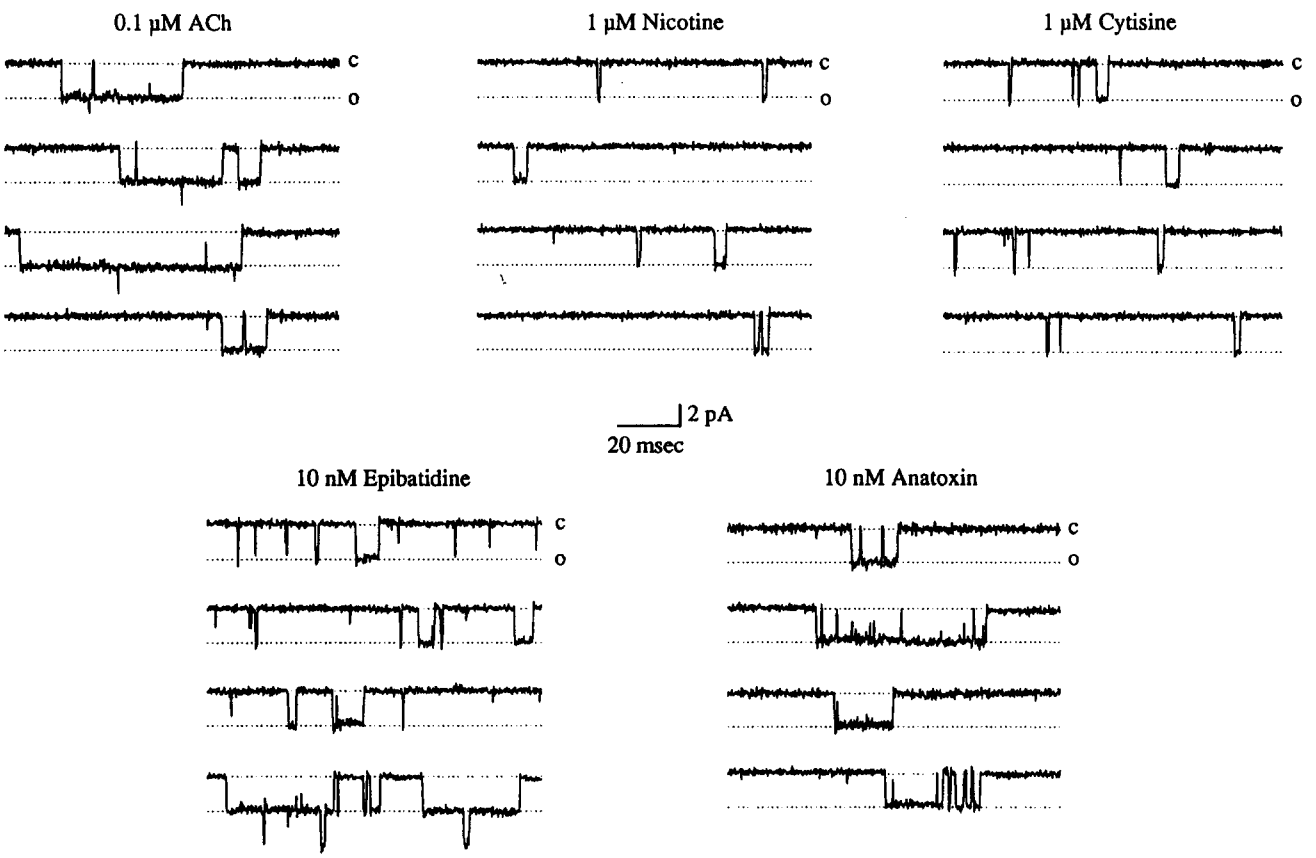

Fig. 3. Representative single-channel responses obtained in outside-out patches for acetylcholine (0.1 μM), nicotine (1 μM), cytisine (1 μM), epibatidine (10 nM) and anatoxin (10 nM). The data were filtered at 2.5 kHz. O, open channel; C, closed channel. Membrane potential −70 mV.

observed on application of tubocurare to outside-out patches (Fig. 5). No channels were observed with tubocurare in outside-out patches from oocytes incubated for 1 h in 3 μM α-bungarotoxin, or from non-injected oocytes. The conductance of the channels activated by tubocurare was found to be 40.2 ± 1.9 pS, close to the values obtained with other agonists. At high tubocurare concentrations and high negative membrane potentials, a second conductance level of about 10 pS was observed. These results clearly indicate an agonist activity of tubocurare on the fetal rat muscle nicotinic acetylcholine receptor. The open-channel lifetime of the single channels activated by tubocurare was very brief and could be fitted at −110 mV with a single exponential distribution with a time constant of 0.33 ± 0.09 ms (average of 5 patches).

Single-channel currents were also observed on application of methyllycaconitine, another antagonist of the nicotinic acetylcholine receptor, to outside-out patches (Fig. 5). Again, no channel events occurred in patches from oocytes incubated in 3 μM α-bungarotoxin for 1 h. However, except in outside-out patches containing a large number of the rat muscle nicotinic acetylcholine receptors, these channels were only seen infrequently and therefore not investigated further.

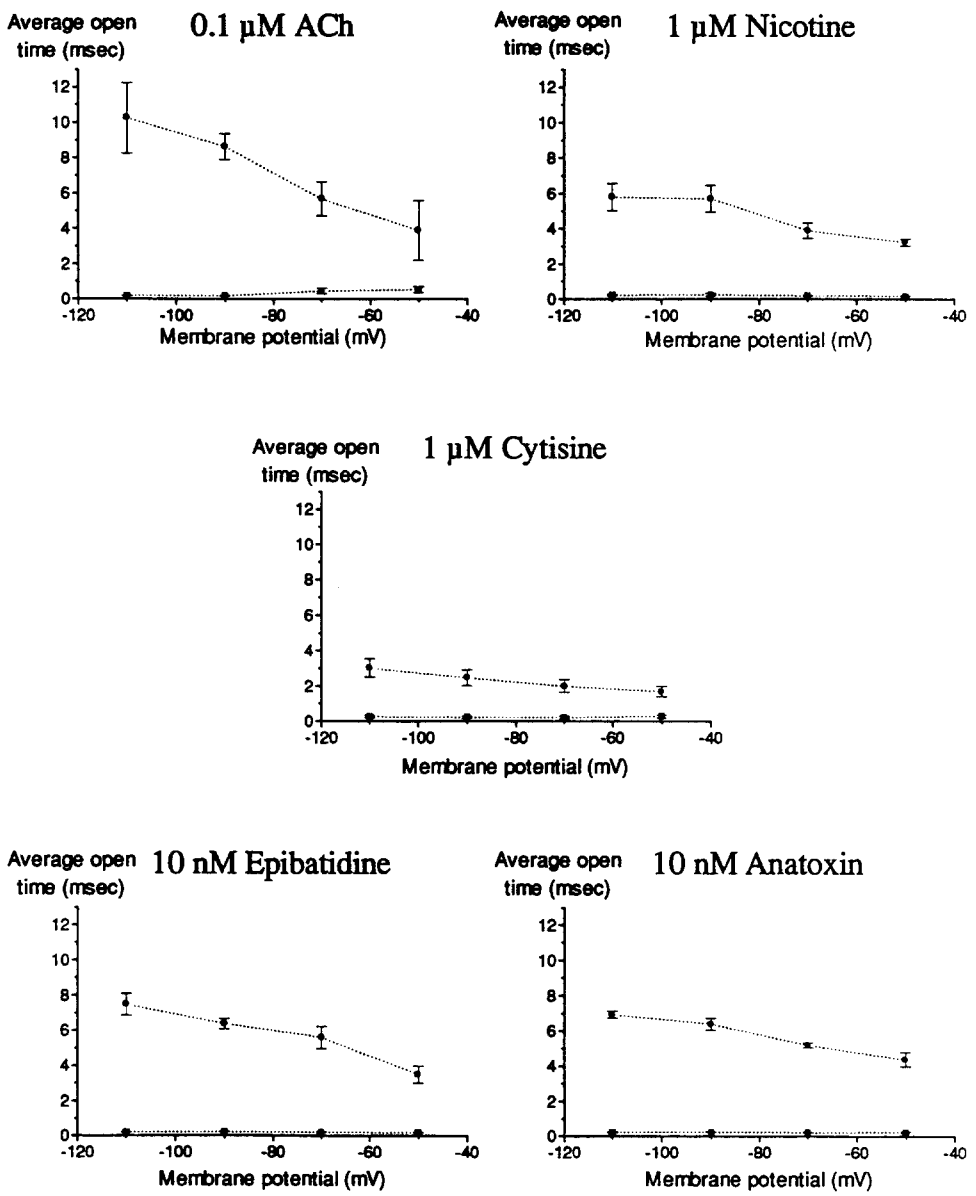

Fig. 4. Dependence of time constants on membrane potential for single channels with acetylcholine (0.1 μM), nicotine (1 μM), cytisine (1 μM), epibatidine (10 nM) or anatoxin (10 nM) as agonist. Open channels were considered terminated when the amplitude decreased to below 50% of the mean amplitude. Time constants were determined by maximum likelihood fitting of two exponentials to binned histograms (bin width 0.1 ms), with no correction for missed events. τ1 (short component) ■, τ2 (long component) ●. Each point is the average of at least three patches.

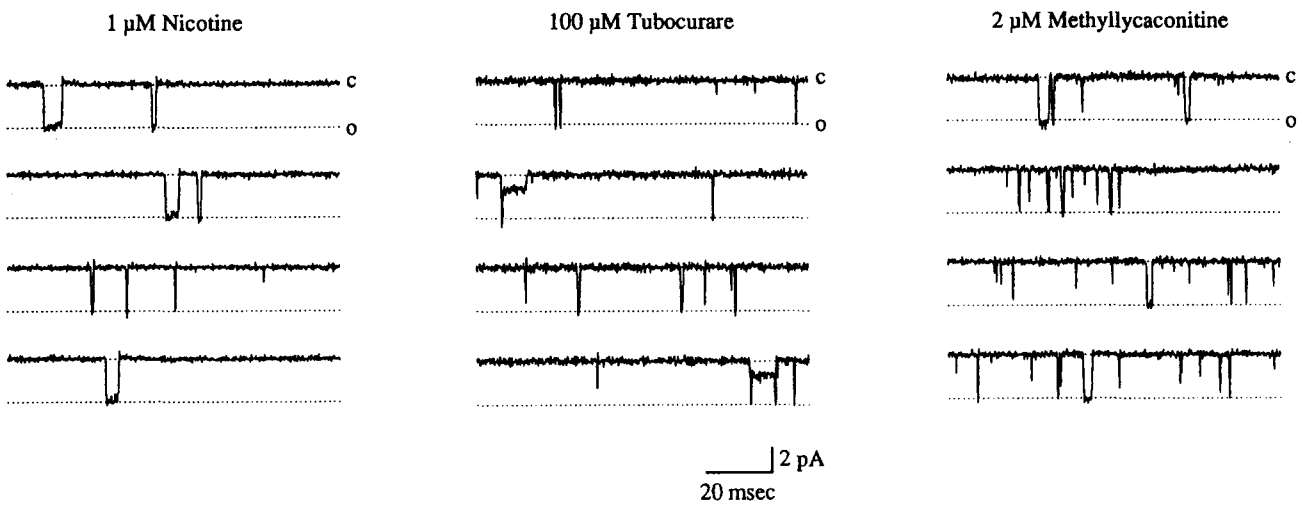

Fig. 5. Single-channel currents activated by nicotine (1 μM), tubocurare (100 μM) and methyllycaconitine (2 μM) in outside-out patches. The data were filtered at 2.5 kHz. O, open channel, C, closed channel. Membrane potential −70 mV

3.2.3. Physostigmine, galanthamine and their methyl derivatives

Recently, physostigmine, galanthamine and related substances have been reported as a new class of agonists, acting at a site on the nicotinic acetylcholine receptor separate from acetylcholine, on nicotinic acetylcholine receptors from adult frog muscle (Shaw et al., 1985), neurons (Albuquerque et al., 1991; Pereira et al.,

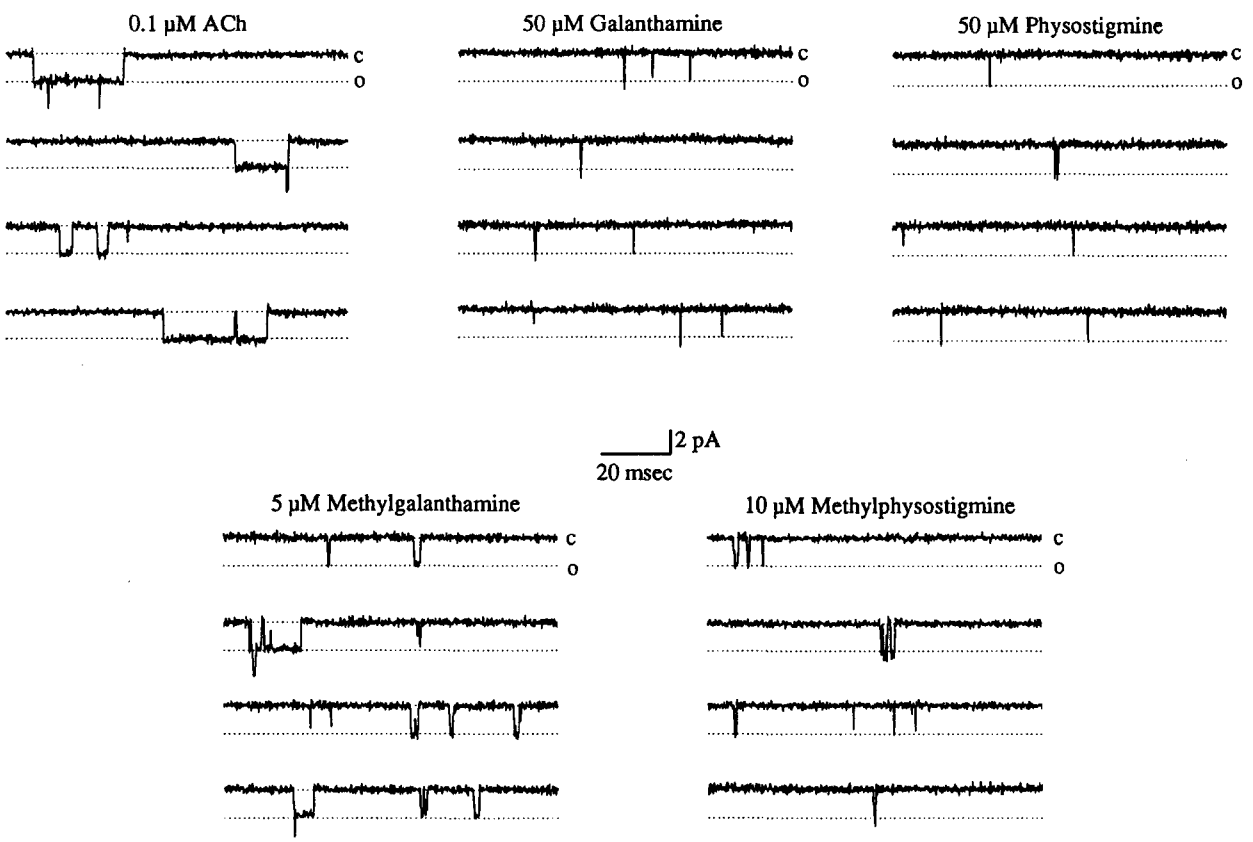

Fig. 6. Typical single-channel currents obtained from outside-out patches for acetylcholine (0.1 μM), physostigmine (50 μM), galanthamine (50 μM), methylphysostigmine (10 μM) and methylgalanthamine (5 μM). The data were filtered at 2.5 kHz. O, open channel, C, closed channel. Membrane potential −50 mV.

Table 3
Time constants fitted to the open-time distribution in outside-out patches for different agonists

Substance	τ1	τ2	% τ1	% τ2
10 nM Epibatidine	0.20±0.01	7.46±0.62	84±2	16±2
10 nM Anatoxin	0.22±0.02	6.95±0.21	65±1	35±1
0.1 μM Acetylcholine	0.18±0.03	10.25±1.98	79±5	21±5
1 μM Nicotine	0.23±0.03	6.03±0.84	85±12	15±12
1 μM Cytisine	0.26±0.06	3.02±0.52	61±12	39±12

Open channels were considered terminated when the amplitude decreased to below 50% of the mean amplitude. Time constants were determined by maximum likelihood fitting of two exponentials to binned histograms (bin width 0.1 ms), with no correction for missed events. τ1, short component, τ2, long component. Each point is the average of at least 3 patches. Since the data were filtered at 2.5 kHz, the τ1 values are probably limited by the electronics. Membrane potential −110 mV.

Table 4
Single-channel conductance measured with methylgalanthamine or methylphysostigmine as agonist

Substance concentration (μM)	Conductance (pS)
Methylgalanthamine	
10	38.1±1.7
50	38.0±1.8
100	39.4±1.8
Methylphysostigmine	
10	36.2±1.9
50	41.9±2.2
100	41.0±2.3

Conductances were evaluated from the slopes of *i-V* plots of amplitude (average of at least 3 patches) against membrane potential.

1993a,b,1994), and PC12 cells (Storch et al., 1995). Fig. 6 shows typical examples of the single-channel currents observed on application of physostigmine, methylphysostigmine, galanthamine or methylgalanthamine to outside-out patches. Such channel events were never seen in patches from non-injected oocytes, suggesting that the responses result from an agonistic activity of these substances on the muscle nicotinic acetylcholine receptor. As estimated from the frequency of channel openings, the rank order of activity for these drugs was methylgalanthamine > methylphysostigmine ≫ galanthamine ≥ physostigmine.

The single-channel conductances obtained for methylphysostigmine or methylgalanthamine are given in Table 4. These values agree well with those obtained using acetylcholine or other classical nicotinic agonists.

The single-channel events induced by physostigmine and galanthamine were too short to be resolved at the

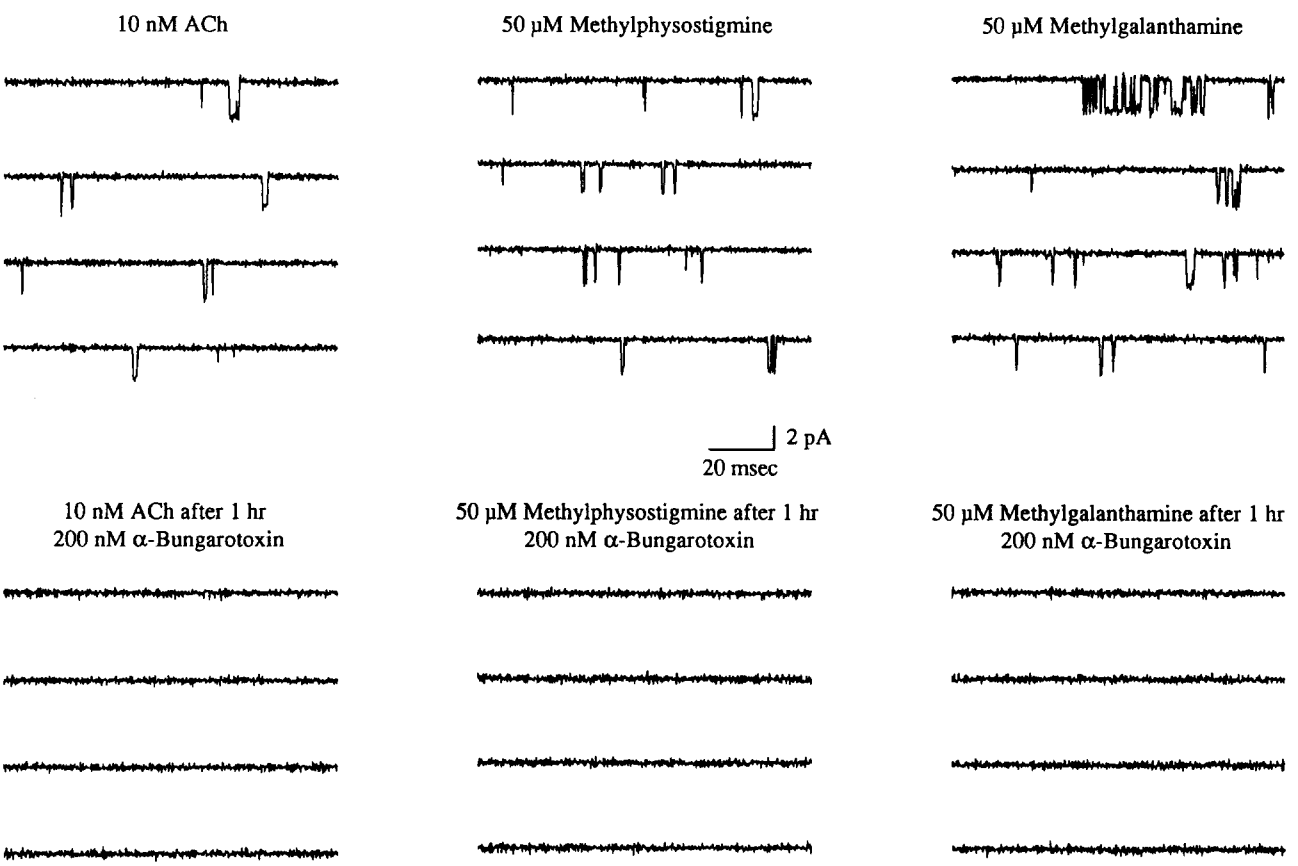

Fig. 7. Effect of oocyte incubation in α-bungarotoxin. Responses are shown to acetylcholine (10 nM), methylphysostigmine (50 μM) and methylgalanthamine (50 μM) in outside-out patches excised before (above), and after (below) incubation of the oocyte for 1 h in 200 nM α-bungarotoxin. Membrane potential −50 mV.

Table 5
Time constants fitted to the open-time distribution for methylphysostigmine or methylgalanthamine

Substance	τ1 (ms)	τ2 (ms)
Methylgalanthamine		
1 μM	0.22 ± 0.03	4.18 ± 0.66
5 μM	0.28 ± 0.06	4.04 ± 0.51
10 μM	0.16 ± 0.02	1.54 ± 0.84
Methylphysostigmine		
10 μM	0.12 ± 0.01	1.36 ± 0.70

Open channels were considered terminated when the amplitude decreased to below 50% of the mean amplitude. Time constants were determined by maximum likelihood fitting of two exponentials to binned histograms (bin width 0.1 ms), with no correction for missed events. τ1, short component, τ2, long component. The τ1 values are probably limited by the 2.5 kHz filter. Each value is the average of at least 3 patches. Membrane potential −110 mV.

2.5-kHz filter setting, so that only methylphysostigmine and methylgalanthamine were used for the determination of open-channel lifetimes.

Table 5 shows the time constants obtained for open times at −110 mV at low concentrations of methylgalanthamine and methylphysostigmine. Two components could be fitted and the value of the longer time constant decreased with increasing concentration. At 50 μM or 100 μM methylgalanthamine or methylphysostigmine, the longer class of open times was observed only in a few patches and then yielded time constants around 1 ms. In most patches the mean open time observed was between 0.1 and 0.3 ms for these concentrations of methylgalanthamine. Since physostigmine and galanthamine are known to be open-channel blockers of the nicotinic acetylcholine receptor (Albuquerque et al., 1991; Pereira et al., 1993b), it is likely that the decrease in channel open time reflects this property. Ideally one would like to measure the open time at concentrations below 1 μM, however the frequency of channel opening then becomes very low.

The probability of channel opening (P_0) was calculated for 100 μM methylgalanthamine ($P_{0,mg}$) in 3 patches at −50 mV, and compared to that with 100 μM tubocurare as agonist ($P_{0,tc}$); single channels observed on application of 100 μM tubocurare have a similar mean open time to those activated by 100 μM methylgalanthamine. The open probability was similar for both agonists: patch 1 $P_{0,mg} = 0.035$, $P_{0,tc} = 0.040$; patch 2 $P_{0,mg} = 0.005$, $P_{0,tc} = 0.007$; patch 3 $P_{0,mg} = 0.032$, $P_{0,tc} = 0.025$.

In contrast to the classical agonists, no clear trend of the mean open time with membrane potential could be observed for methylphysostigmine and methylgalanthamine.

Surprisingly, the single-channel currents induced by physostigmine, galanthamine and their derivatives were not observed in outside-out patches from oocytes that had been incubated for 1 h in 200 nM α-bungarotoxin (Fig. 7). Also, the frequency of single channels activated by these drugs was dramatically reduced in the presence of mecamylamine, or methyllycaconitine, a well-known competitive nicotinic acetylcholine receptor antagonist (not shown). This also appeared to be the case for methyllycaconitine (not shown). However, the effect due to methyllycaconitine was partially obscured because this blocker itself can activate some nicotinic acetylcholine receptor channels as described above.

4. Discussion

Using the voltage-clamp and patch-clamp methods, we have investigated the activity of a range of agonists on the fetal rat muscle nicotinic acetylcholine receptor, expressed in *Xenopus* oocytes. The agonist activity of the substances, as measured with the voltage-clamp method on whole oocytes, varied over several orders of magnitude in the sequence anatoxin ≥ epibatidine > acetylcholine > DMPP ≫ cytisine > pyrantel > nicotine > coniine > tubocurare > lobeline.

These compounds were chosen, from the large number of known nicotinic agonists, to represent a set of compact, sterically defined ligands of differing structures and with a range of agonist potencies. Anatoxin (Spivak et al., 1980; Swanson et al., 1986) has been reported to be a powerful agonist for neuromuscular nicotinic acetylcholine receptors. More recently, epibatidine has been described as a nicotinic analgesic (Qian et al., 1993) and as an agonist of human muscle type nicotinic acetylcholine receptor approximately 100 times more potent than nicotine in ion flux studies on TE671 cells (Badio and Daly, 1994). Our electrophysiological results on *Xenopus* oocytes expressing the fetal rat muscle nicotinic acetylcholine receptor clearly show that both epibatidine and anatoxin are potent agonists of the receptor, being rather more potent than acetylcholine and indeed the most effective agonists of all the substances that we have tested.

Using this set of different agonists, we performed a molecular modelling study as a step towards elucidation of the structure-activity relationship of these substances. Superposition of individual agonist structures on that of anatoxin, which has the highest activity of the selected agonists, does show structural features that contribute to their relative activity. The model showed a good correlation between the steric and electrostatic properties calculated for this group of structurally diverse agonists and their relative agonist activity. This suggests that for a more comprehensive set of active compounds, a more systematic approach, such as a CoMFA (Comparative Molecular Field Analysis) study, may be feasible.

More precise information about the action of agonists on ion channels can be obtained from single channel experiments, which were performed for acetylcholine, nicotine, cytisine, epibatidine and anatoxin in this study. Single channels were observed for all of these agonists in outside-out patches from oocytes and these were blocked

by prior incubation of the oocyte in α-bungarotoxin. The values of the single-channel conductances were about the same, just as would be expected if these agonists all act upon the same nicotinic receptor.

The value of 36.8 ± 0.5 pS obtained for the single-channel conductance agrees well with previous reports for nicotinic acetylcholine receptor from embryonic rat muscle (Witzemann et al., 1990) and denervated adult muscle (Methfessel et al., 1986), expressed in *Xenopus* oocytes. In our investigations, only one conductance class was observed for the agonists listed.

The open-channel lifetimes for the agonists could be fitted with two time constants, of which the longer time constant τ2 was clearly dependent on membrane potential. The shorter time constant τ1 was not sufficiently resolved to enable any conclusions about potential dependence. A strong dependence of τ2 on potential was observed for acetylcholine and epibatidine, whereas for cytisine this dependence was less pronounced (Fig. 4). This voltage dependence of the open-channel lifetime is a property of nicotinic acetylcholine receptor channels containing a mammalian δ subunit (Sakmann et al., 1985). The short component was present for all of the agonists, but was not sufficiently resolved for a detailed characterisation.

The longer time constants τ2 for acetylcholine can be compared to published values for calf muscle nicotinic acetylcholine receptor in *Xenopus* oocytes of 10.2 ± 1.4 ms at −100 mV, 12.5 ± 3.8 ms at −100 mV for calf muscle fibres (Mishina et al., 1986), and 10.2 ± 4.9 ms for mouse muscle nicotinic acetylcholine receptor expressed in *Xenopus* oocytes (Kullberg et al., 1990).

Although the longer component of the mean open times was characteristic of the types of agonists, there is no obvious correlation with their agonistic activity. Thus, the longest openings were induced by acetylcholine, whereas epibatidine and anatoxin were much stronger agonists.

The well-known antagonists of the nicotinic acetylcholine receptor, tubocurare and methyllycaconitine, also induced single channels in outside-out patches. These channels were again absent in patches from oocytes incubated in α-bungarotoxin, and the channel conductances were similar to those obtained with acetylcholine as agonist. Therefore, tubocurare, and possibly also methyllycaconitine, should more correctly be classified as partial agonists of the nicotinic acetylcholine receptor from fetal rat muscle. Curare-induced depolarisation was first reported in embryonic rat muscle (Ziskind and Dennis, 1978), and single-channel currents induced by tubocurare have been observed in human muscle (Jackson et al., 1982), embryonic rat muscle (Morris et al., 1982) and in rat myotubes (Trautmann, 1982). Our results agree particularly well with observations on rat myotubes (Takeda and Trautmann, 1984), where a main conductance of 40 pS as well as a smaller 13 pS channel was reported. The frequency of the small conductance state increased with tubocurare concentration and with hyperpolarisation and was attributed to the dual action of tubocurare as agonist and as channel blocker. Accordingly, of two open-channel time constants observed at low concentrations (τ1 = 0.3 ms, τ2 = 1.7ms at +30 mV, cell attached), the longer one became shorter and finally disappeared at progressively higher concentrations.

Single channels were also observed on application of physostigmine, galanthamine and their methyl derivatives to outside-out patches, and these channels were absent from patches excised from non-injected oocytes. The conductance values agree well with those obtained using acetylcholine or other classic nicotinic agonists. There was no obvious effect of the drug concentration on the single-channel conductance values for methylphysostigmine and methylgalanthamine. This result differs from a report that the conductance of channels induced by physostigmine in frog muscle at 10°C decreased from 29.0 pS for 0.5 μM physostigmine to 18.0 pS for 50 μM physostigmine (Shaw et al., 1985), in accordance with the open-channel blocking properties of physostigmine. Instead, our findings suggest that for the methylated compounds, the open-channel block manifests itself as a reduction of the open-channel lifetime with increasing drug concentrations, starting at about 10 μM for methylgalanthamine. One interpretation of this finding could be that the blocked state of the open channel induced by these compounds is long-lived in the fetal rat muscle nicotinic receptor and short-lived in other preparations.

No responses to physostigmine or methylgalanthamine were seen on application to oocytes in the two electrode whole-cell recording mode. This is inconsistent with the data from outside-out patches, where appreciable activity of single nicotinic acetylcholine receptor channel currents was activated by these drugs. However, the absence of whole-cell responses has also been reported in cell lines (Storch et al., 1995; Pereira et al., 1994) and hippocampal neurons (Pereira et al., 1993a,b), where these compounds also activated nicotinic acetylcholine receptor currents in outside-out patches. The reason for this discrepancy has not yet been established, but explanations have been proposed (Pereira et al., 1994). Thus, at drug concentrations sufficient for whole-cell current responses, the open-channel block may outweigh the receptor activation. Alternatively, the frequency and duration of channel openings may simply not be sufficient to yield a measurable whole-cell current response in cell lines and neurons. However, for fetal rat muscle nicotinic acetylcholine receptor in oocytes, the frequency of single-channel events activated by methylgalanthamine in outside-out patches was appreciable and indeed the open probability was comparable to that elicited by the partial agonist, tubocurare. Yet, tubocurare produced a clear inward current in the whole-cell voltage-clamp and methylgalanthamine did not.

In our experiments, the single-channel current responses to physostigmine were clearly inhibited by mecamylamine and methyllycaconitine as well as by pre-incubation with

α-bungarotoxin. This contrasts with neuronal nicotinic acetylcholine receptors, where classical competitive nicotinic acetylcholine receptor antagonists did not interefere with channel activation by non-competitive agonists (Albuquerque et al., 1991; Pereira et al., 1993a; Storch et al., 1995). This may point to an important difference between the muscle and neuronal forms of nicotinic receptors, possibly indicating that the binding site for classical antagonists partially or completely overlaps the non-competitive agonist binding site in muscle receptors. However, at least for the special case of the fetal rat muscle nicotinic acetylcholine receptor, we cannot exclude that non-competitive agonists, such as physostigmine, may also interact, directly or indirectly, with the classical nicotinic agonist binding site.

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References

Albuquerque, E.X., Y. Aracava, W.M. Cintra, A. Brossi, B. Schönenberger and S.S. Deshpande, 1988, Structure-activity relationship of reversible cholinesterase inhibitors: activation, channel blockade and stereospecificity of the nicotinic acetylcholine receptor-ion channel complex, Braz. J. Med. Bio. Res. 21, 1173.

Albuquerque, E.X., A. Maelicke and E.F.R. Pereira, 1991, (−)Physostigmine directly activates nicotinic acetylcholine receptors from electric tissue, muscle and brain, Soc. Neurosci. Abstr. 17, 585.

Allen, F.H., J.E. Davies, J.J. Galloy, O. Johnson, O. Kennard, C.F. Macrae, E.M. Mitchell, G.F. Mitchell, J.M. Smith and D.G. Watson, 1991, The Cambridge Structural Database System, J. Chem. Inf. Comp. Sci. 31, 187.

Badio, B. and J.W. Daly, 1994, Epibatidine, a potent analgetic and nicotinic agonist, Mol. Pharmacol. 45, 563.

Bormann, J., 1992, U-tube drug application, in: Practical Electrophysiology Methods, eds. H. Kettenmann and R. Grantyn (Wiley-Liss, New York) p. 136.

Colquhoun, D. and B. Sakmann, 1985, Fast events in single-channel currents activated by acetylcholine and its analogues at the frog muscle end plate, J. Physiol. 369, 501.

Jackson, M.B., H. Lecar, V. Askanas and W.K. Engel, 1982, Single cholinergic receptor channel currents in cultured human muscle, J. Neurosci. 2, 1465.

Kullberg, R., J.L. Owens, P. Comacho, G. Mandel and P. Brehm, 1990, Multiple conductance classes of mouse nicotinic acetylcholine receptors expressed in *Xenopus* oocytes, Proc. Natl. Acad. Sci. USA 87, 2067.

Methfessel, C., V. Witzemann, T. Takahashi, M. Mishina, S. Numa and B. Sakmann, 1986, Patch clamp measurements on *Xenopus laevis* oocytes: currents through endogenous channels and implanted acetylcholine receptors and sodium channels, Pflügers Arch. 407, 577.

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

Morris, C.E., M.B. Jackson, H. Lecar, B.S. Wong and C.N. Christian, 1982, Activation of individual acetylcholine channels by curare in embryonic rat muscle, Biophys. J. 37, 19a.

Pereira E.F.R., M. Alkondon, T. Tano, N.G. Castro, M.M. Fróes-Ferrão, R. Rozental, R.S. Aronstam, A. Schrattenholz, A. Maelicke and E.X. Albuquerque, 1993a, A novel agonist binding site on nicotinic acetylcholine receptors, J. Rec. Res. 13 (1-4), 413.

Pereira, E.F.R., S. Reinhardt-Maelicke, A. Schrattenholz, A. Maelicke and E.X. Albuquerque, 1993b, Identification and functional characterization of a new agonist site on nicotinic acetylcholine receptors of cultured hippocampal neurons, J. Pharmacol. Exp. Ther. 256, 1474.

Pereira, E.F.R., M. Alkondon, S. Reinhardt, A. Maelicke, X. Peng, J. Lindstrom, P. Whiting and E.X. Albuquerque, 1994, Physostigmine and galanthamine characterize the presence of the novel binding site on the α4β2 subtype of neuronal nicotinic acetylcholine receptors stably expressed in fibroblast cells, J. Pharmacol. Exp. Ther. 270, 768.

Qian, C., T. Li, T.Y. Shen, L. Libertine, Garahan, J. Eckman, T. Biftu and S. Ip, 1993, Epibatidine is a nicotinic analgesic, Eur. J. Pharmacol. 250, R13.

Sakmann, B., C. Methfessel, M. Mishina, T. Takahashi, T. Takai, M. Kurasaki, K. Fukuda and S. Numa, 1985, Role of acetylcholine receptor subunits in gating of the channel, Nature (London) 318, 538.

Shaw, K.-P., Y. Aracava, A. Akaike, J.W. Daly, D.L. Rickett and E.X. Albuquerque, 1985, The reversible cholinesterase inhibitor physostigmine has channel-blocking and agonist effects on the acetylcholine receptor-ion channel complex, Mol. Pharmacol. 28, 527.

Spitzer, K.W. and J.H. Bridge, 1989, A simple device for rapidly exchanging solution surrounding a single cardiac cell, J. Am. Physiol. 256, C441.

Spivak, C.E., B. Witkop and E.X. Albuquerque, 1980, Anatoxin-a: A novel, potent agonist at the nicotinic receptor, Mol. Pharmacol. 18, 384.

Storch, A., A. Schrattenholz, J.C. Cooper, E.M.A. Ghani, O. Gutbrod, K.-H. Weber, S. Reinhardt, C. Lobron, B. Hermsen, V. Soskiç, A. Schrattenholz, E.F.R. Pereira, E.X. Albuquerque, C. Methfessel and A. Maelicke, 1995, Physostigmine, galanthamine and codeine act as 'noncompetitive nicotinic receptor agonists' on clonal rat pheochromocytoma cells, Eur. J. Pharmacol. Mol. Pharmacol. Sect. 290, 207.

Swanson, K.L., C.N. Allen, R.S. Aronstam, H. Rapoport and E.X. Albuquerque, 1986, Molecular mechanisms of the potent and stereospecific nicotinic receptor agonist (+)-anatoxin-a, Mol. Pharmacol. 29, 250.

Takeda, K. and A. Trautmann, 1984, A patch-clamp study of the partial agonist actions of tubocurare on rat myotubes, J. Physiol. 349, 353.

Trautmann, A., 1982, Curare can open and block ionic channels associated with cholinergic receptors, Nature (London) 276, 622.

Witzemann, V., E. Stein, B. Barg, T. Konno, M. Koenen, W. Kues, M. Criado, M. Hoffmann and B. Sakmann, 1990, Primary structure and functional expression of the α-, β-, γ-, δ- and ϵ-subunits of the acetylcholine receptor from rat muscle, Eur. J. Biochem. 194, 437.

Ziskind, L. and M.J. Dennis, 1978, Depolarising effect of curare on embryonic rat muscles, Nature (London) 276, 622.