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Mechanism of neuromuscular blockade induced by phenthonium, a quaternary derivative of (−)-hyoscyamine, in skeletal muscles

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1 The mechanisms underlying the postjunctional blockade induced by phenthonium [N-(4-phenyl) phenacyl 1-hyoscyamine] were investigated in mammalian and amphibian muscles. This muscarinic antagonist was previously shown to enhance specifically the spontaneous acetylcholine (ACh) release at concentrations that blocked neuromuscular transmission.

2 In both rat diaphragm and frog sartorius muscles, phenthonium (Phen, 1–100 μM) depressed the muscle twitches elicited by nerve stimulation (IC_{50}: 23 μM and 5 μM, respectively), and blocked the nerve-evoked muscle action potential. The neuromuscular blockade was not reversed after incubation with neostigmine.

3 Equal concentrations of Phen decreased the rate of rise and prolonged the falling phase of the directly elicited action potential in frog sartorius muscle fibres, indicating that the drug also affects the sodium and potassium conductance.

4 Phen (50 and 100 μM) protected the ACh receptor against α-bungarotoxin (BUTX) blockade in the mouse diaphragm allowing recording of endplate potentials and action potentials after 5 h wash with physiological salt solution.

5 Phen (10–100 μM) produced a concentration- and voltage-dependent decrease of the endplate current (e.p.c.), and induced nonlinearity of the current-voltage relationship. At high concentrations Phen also shortened the decay time constant of e.p.c ($\tau_{e.p.c.}$) and reduced its voltage sensitivity.

6 At the same range of concentrations, Phen also reduced the initial rate of [^{125}I]-BUTX binding to junctional ACh receptors of the rat diaphragm (apparent dissociation constant = 24 μM), the relationship between the degree of inhibition and antagonist concentration being that expected for a competitive mechanism.

7 It is concluded that Phen affects the electrical excitability of the muscle fibre membrane, and blocks neuromuscular transmission through a mechanism that affects the agonist binding to its recognition site and ionic channel conductance of the nicotinic ACh receptor.

Keywords: Nicotinic receptor; noncompetitive blocker; ionic channel; endplate current; muscarinic antagonist

Introduction

Phenthonium [N-(4-phenyl) phenacyl l-hyoscyamine] is a quaternary derivative of (−)-hyoscyamine and a competitive muscarinic antagonist 100 times less potent than atropine (Souccar *et al*., 1994). In the rat diaphragm muscle, phenthonium increased the spontaneous acetylcholine (ACh) release without affecting that induced by nerve stimulation, and blocked the indirectly-elicited muscle twitch (Fann *et al*., 1990; Cysneiros *et al*., 1991; Souccar *et al*., 1994). The prejunctional effect of phenthonium was unaccompanied by membrane depolarization or blockade of the acetylcholinesterase activity, and was unrelated to the temperature or to an increased entry of Ca^{2+} into the nerve terminal (Fann *et al*., 1990). These observations suggested a possible interaction of phenthonium with inhibitory muscarinic cholinoceptors located on the motor nerve terminal (Wessler, 1989; Bowman *et al*., 1990), as a mechanism accounting for the enhanced spontaneous ACh release. However, the facilitatory effect of phenthonium was not reproduced or antagonized by the muscarinic antagonists atropine, methylatropine or pirenzepine (Fann *et al*., 1990; Souccar *et al*., 1994). The prejunctional effect of phenthonium, on the other hand, was obtained at concentrations that also produced postjunctional blockade (Fann *et al*., 1990).

This study investigated the mechanisms underlying the postjunctional blockade induced by phenthonium at the neuromuscular junction. The results obtained from functional, electrophysiological and binding experiments indicated that this muscarinic antagonist affects the ACh binding to its recognition site and decreases the conductance of the nicotinic receptor/ionic channel.

Methods

The experiments were carried out on the phrenic nerve-diaphragm muscle preparations from male rats (Wistar 200–250 g) and mice (albino Swiss 25–30 g). In a few experiments the rat levator ani and frog (*Rana catesbeiana* 200–250 g) sartorius muscles were used. Rats and mice were killed by ether anaesthesia and exsanguination through the abdominal aorta. Frogs were killed by double pithing. The muscles and respective nerves were rapidly isolated and kept in physiological solutions with the following composition: Tyrode solution (in mM); NaCl 135, $NaHCO_3$ 15, KCl 5, $CaCl_2$ 2, $MgCl_2$ 1, NaH_2PO_4 1 and glucose 11; pH 7.3–7.4 after gassing with 95% O_2 + 5% CO_2 and frog Ringer solution (in mM): NaCl 115.5, KCl 2.0, $CaCl_2$ 1.8, NaH_2PO_4 0.7, Na_2HPO_4 1.3 and glucose 11.1, pH 6.9.

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Tension recordings

The phrenic nerve-hemidiaphragm or sciatic nerve-sartorius muscles were mounted under 2 g tension in organ baths containing 10 ml of physiological solution continuously gassed with 95% O_2 + 5% CO_2. Muscle twitches were elicited by supramaximal pulses alternatively applied to the nerve (0.5 ms, 0.1 Hz) and the muscle fibres (2 ms, 0.1 Hz) through bipolar platinum electrodes immersed in the bath. Muscle twitches were isometrically recorded using a force-displacement transducer (FT03, Grass Instruments) on a polygraph (Beckman R116). After 30 min stabilization, the resting tension was readjusted and the effects of single concentrations of phenthonium (Phen, 1–100 μM) were recorded for 30 min. The IC_{50} (median inhibitory concentration) values were determined from concentration-effect relationships as the concentrations that produced 50% inhibition of maximum contraction.

For the receptor protection studies the nerve-elicited muscle twitches of the mouse diaphragm were recorded as detailed elsewhere (Lapa *et al*., 1974). Muscle preparations were mounted in organ baths as described above, and after 30 min equilibration they were incubated with either Phen (10–100 μM) or (+)-tubocurarine (TC, 3–75 μM). After 30 min, α-bungarotoxin (BUTX–0.25 μM) was added to the preparations in the presence of either drug. Washout of the drugs was done after 30 min with a continuous flow of Tyrode solution containing TC (100 μM) for 30 min, followed by the nutritive solution alone for 2 to 8 h. Following different intervals of washout, the muscles were transferred to a chamber for intracellular recordings as described below. Control preparations were incubated with either Phen (100 μM) or TC (100 μM) for 60 min, after which they were rinsed with Tyrode solution containing TC (100 μM), for 30 min, followed by Tyrode solution alone for 2 to 8 h. Amplitudes of the muscle twitches were measured and expressed as percentage of the control recorded immediately before addition of the drug.

Intracellular studies

The muscle preparation was pinned down slightly stretched on a Sylgard (Dow Corning) base in a 10 ml chamber and continuously perfused with nutritive solution. Endplate potentials (e.p.p.) and nerve-induced muscle action potentials were intracellularly recorded with standard glass microelectrodes (22–24°C) as described before (Fann *et al*., 1990). The directly elicited action potentials were recorded from glycerol-shocked (600 mM) sartorius muscles (Gage & Eisenberg, 1967) by passing a 30 ms depolarizing pulse through a microelectrode inserted 50 μm away from the recording electrode (Souccar *et al*., 1984). The maximal rate of rise and fall of action potentials were measured using an RC circuit (100 kOhm, 100 pF). Endplate currents (e.p.cs) were recorded in 'cut-muscle' preparations of the rat levator-ani using the two microelectrodes voltage-clamp technique (Takeuchi & Takeuchi, 1959) as detailed elsewhere (Souccar *et al*., 1984; 1991). This preparation was previously described by Souccar *et al*., (1982, 1991) and used in these experiments because it presented less nerve conduction blockade than the diaphragm muscle (Glavinovic, 1979). After 10–15 min transection the resting membrane potential of the muscle fibres ranged between −50 and −40 mV. Voltage recording and current passing electrodes had resistances of 5–10 MOhm. E.p.cs were elicited by stimulating the nerve at 0.2 Hz with supramaximal pulses (0.05 ms duration) through a bipolar platinum electrode and a Grass S88 stimulator coupled to a SIU5 stimulus isolation unit (Grass Instruments). The membrane potential was held at −50 mV and e.p.cs were elicited at 10 mV steps between −110 mV to +50 mV, using an Axoclamp-2A voltage-clamp circuit (Axon Instruments). Currents and voltage signals were amplified, displayed on a Tektronix oscilloscope and recorded on either moving 35 mm film by a Grass camera or digitized at 10 kHz and stored on an IBM-AT computer. In the latter case a TL-1 Labmaster system connected to a digital interface board (Scientific Solutions) was used. Amplitudes and decay of the currents were measured manually or using the PClamp programme (Axon Instruments). The time constant of e.p.c. decay ($\tau_{e.p.c.}$) was determined from the exponential decay of e.p.c. between 20 and 80% of the peak amplitude.

Binding studies

Rat diaphragm muscles were kept in cold nutritive solution (4°C) and dissected in junctional and extrajunctional strips (Smith & Chapman, 1987). Samples of junctional (20 mg) regions were prepared in 1 ml physiological solution containing bovine serum albumin (0.5%) and incubated in triplicate to final concentrations of 0.5–3 nM [^{125}I]-BUTX for 0.5 to 5 h, at 37°C. Specific binding was determined in samples incubated 1 h before in 500 fold excess unlabelled α-BUTX at 22–24°C. Nonspecific binding ranged between 35% and 40% of the total binding. The effect of Phen (0.1–100 μM) or TC (0.1–25 μM) on the initial rate of toxin binding was tested by incubation of one concentration of each antagonist 30 min before 2 nM [^{125}I]-BUTX for 3 h. The reaction was stopped by addition of 0.25 μM unlabelled toxin and the tissue samples were successively washed in ice-cold physiological solution for 15–18 h. The sample radioactivity was measured on a γ counter (Abbot) and expressed as specific binding in fmol mg^{-1} of muscle tissue. The apparent dissociation constant (K_I) of Phen was determined according to Colquhoun and Rang (1976) using a relationship similar to that for competitive drug antagonism (Schild, 1947): $r-1=x_I/K_I$, where 'r' is the ratio of the rate constant for [^{125}I]-BUTX binding in the absence of inhibitor to that in the presence of inhibitor, and x_I is the concentration of the inhibitor. K_I was determined as the slope of a straight line obtained from a plot of $r-1$ against x_I (Colquhoun & Rang, 1976).

Drugs

Drugs used were: (+)-tubocurarine, α-bungarotoxin, bovine serum albumin (Sigma Chemical Co.), and [^{125}I]-a-bungarotoxin (>200 μCi mmol^{-1}, Amersham Int.). Phenthonium [N- (4-phenyl)-phenacyl l-hyoscyamine] bromide was provided by Dr D. Della Bella from Zambon Laboratories (Milano, Italy). All other reagents were of analytical grade. Stock solutions of phenthonium were prepared in 0.01 N hydrochloric acid (12%) and diluted in Tyrode solution to the needed concentrations.

Statistics

Data are presented as means ± s.e. mean. Differences between data were detected by Student's two tailed t test and considered significant at $P<0.05$.

Results

Muscle twitch and protection studies

In the rat and mouse diaphragm muscles as well as in frog sartorius preparations, Phen (1–100 μM) depressed the nerve-

evoked muscle twitches proportionately to the concentration without affecting the directly elicited muscle twitches. The IC_{50} (50% inhibitory concentration) values determined in rat and frog preparations were 23 μM and 5 μM, respectively. Phen-induced depression of the indirectly elicited muscle twitches was enhanced in the presence of the anticholinesterase agent neostigmine (10 μM), suggesting a noncompetitive blockade of neuromuscular transmission (Figure 1). In parallel experiments, the same concentration of neostigmine reversed the blockade induced by the competitive nicotinic antagonist (+)-tubocurarine (TC, 1.5 μM). However, washing the preparations with nutritive solution, completely reversed the blockade induced by Phen (Figure 1).

Phen was less potent than TC in blocking the neuromuscular transmission of the diaphragm muscle. After 30 min exposure of mouse preparations to 100 μM Phen or 3 μM TC the amplitudes of the nerve-induced muscle twitches were reduced by 95% and 98% of control, respectively (Figure 2). In similar preparations, α-bungarotoxin (BUTX, 0.25 μM) produced a complete and irreversible blockade of the indirectly elicited muscle twitches within 30 min.

Exposure of the diaphragm muscle to Phen (100 μM) 30 min before the addition of BUTX (0.25 μM), and subsequent washing with nutritive solution for 3 h restored the nerve-elicited muscle twitches to 85% of control values (Figure 2). In parallel experiments, previous incubation of a concentration of TC (3 μM) equipotent to that of Phen (100 μM) did not protect the AChR against the toxin blockade. However, protection of the cholinoceptor was effective only after exposure to high concentrations of TC (35 μM and higher) (Figure 2).

Effects on the neuromuscular transmission

In control rat diaphragm muscle fibres, the mean resting membrane potential (RMP) was -82 ± 1 mV (508 fibres in 38 muscles). The nerve-elicited muscle action potential (AP) had an amplitude of 106 ± 1 mV and a time to 50% repolarization of 1.08 ± 0.03 ms; the maximal rates of rise and fall of the AP were respectively, 576.5 ± 15.3 Vs^{-1} and 152.9 ± 6.3 Vs^{-1} (40 cells in 11 muscles). Incubation with Phen (100 μM) did not affect the RMP, whereas the amplitudes of the indirectly elicited muscle APs were progressively depressed and substituted by e.p.ps of 1 to 2 mV amplitude after 10 min (Figure

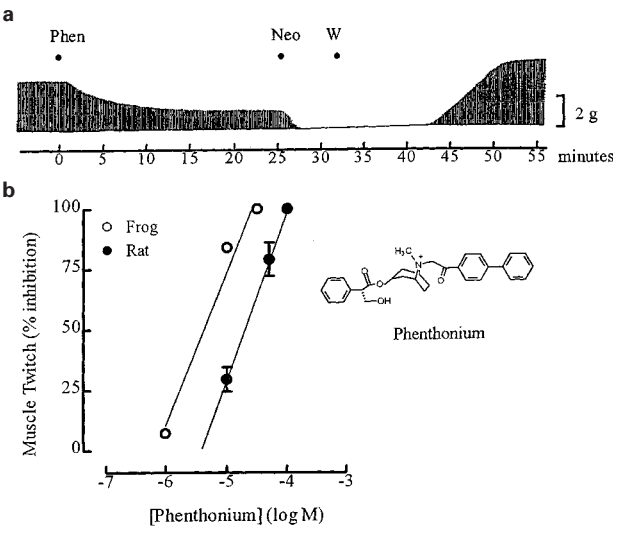

Figure 1 (a)Time-course of the effect of phenthonium (Phen, 20 μM) on muscle twitches of the rat diaphragm induced by nerve stimulation (0.2 Hz). Notice the enhanced blockade of the muscle twitches after incubation of neostigmine (Neo, 10 μM) and its reversal after washout (W) of the drugs. (b) Concentration-effect relationships for the twitch block of frog sartorius and rat diaphragm muscles after, respectively, 15 and 30 min incubation with Phen (chemical structure on the right hand). Muscle twitches were elicited by nerve stimulation and expressed, in ordinates, as percentage inhibition of the maximal twitch amplitude recorded before incubation of the drug. Symbols are means and vertical lines s.e. mean of 6–8 muscles.

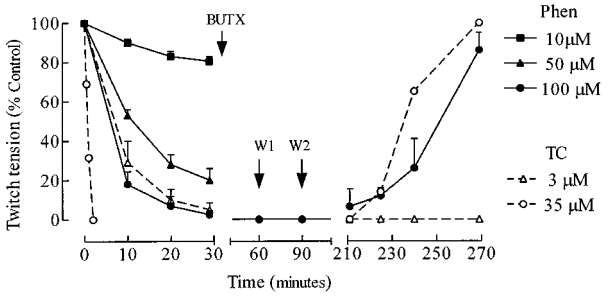

Figure 2 Effects of phenthonium (Phen: 10, 50 and 100 μM) and (+)-tubocurarine (TC: 3 and 35 μM) on the nerve-elicited muscle twitches of the mouse diaphragm. The drugs were incubated at time zero and after 30 min α-bungarotoxin (BUTX, 0.25 μM) was added to the preparations. After further 30 min the drugs were washed out with a solution containing TC (10 μM, W1), for 30 min, followed by Tyrode solution alone (W2). Notice complete recovery of the muscle twitches in preparations previously incubated with Phen (100 μM) and TC (35 μM) after 3 h wash. Muscle twitches were expressed as percentage of the maximal twitch amplitude recorded before incubation of the drugs. Symbols and vertical lines are means and s.e.mean of 4–6 muscles.

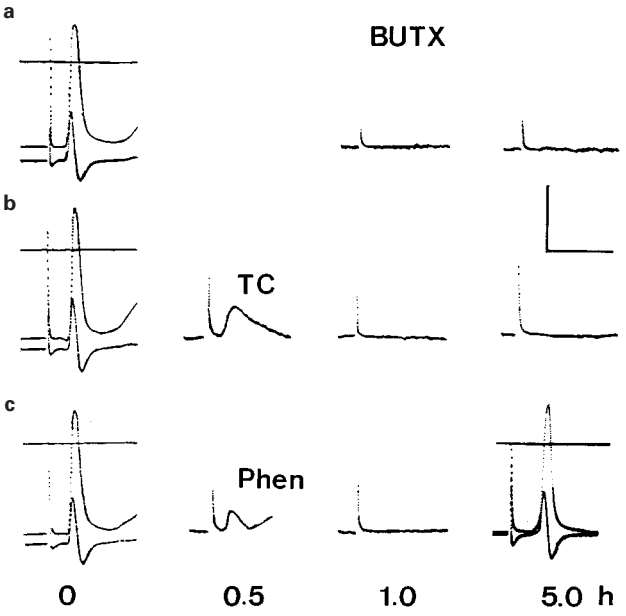

Figure 3 Effects of phenthonium (Phen) and (+)-tubocurarine (TC) on the neuromuscular transmission of the mouse phrenic-nerve diaphragm muscle. Nerve-elicited action potentials recorded intracellularly from superficial cells in control conditions (time zero) were replaced by endplate potentials after 30 min incubation with Phen (100 μM) or TC (5 μM). Incubation of α-bungarotoxin (BUTX, 0.25 μM) alone (a) or in association with either TC (b) or Phen (c) blocked the neuromuscular transmission after 30 min. Washout of the preparations with Tyrode solution containing 100 μM TC (30 min) followed by Tyrode solution (4.5 h) restored the neuromuscular transmission only in those muscles exposed to Phen. At time 0 and 5 h the horizontal lines correspond to zero potential, upper trace is the action potential and lower trace is its first derivative (dv/dt). Calibration: vertical bar is 50 mV for the action potential and 2.5 mV for the endplate potential; horizontal bar is 5 ms.

3). Washing the preparations with nutritive solution completely reversed the neuromuscular blockade after 15 min.

Exposure of similar preparations to BUTX (0.25 μM) blocked irreversibly the e.p.ps after 10 min. Incubation of Phen (50 and 100 μM) before BUTX (0.25 μM) protected the ACh receptor against toxin binding allowing recordings of e.p.ps or AP after 5 h wash (Figure 3). In parallel experiments TC (5 μM) was ineffective in protecting the ACh receptor against BUTX, and after 5 h washout only e.p.ps of 1 to 2 mV amplitudes were recorded in a few cells.

To evaluate the effect of Phen on the electrical excitability of the muscle membrane, APs induced by direct stimulation of the frog sartorius muscle fibres were recorded. Under control conditions the threshold and amplitude of the directly elicited APs were 41.3 ± 1.3 mV and 135.5 ± 1.0 mV, while the half decay time, maximal rates of rise and fall were 0.89 ± 0.02 ms, 900 ± 18 Vs^{-1} and 480 ± 9 Vs^{-1}, respectively. Phen (100 μM) did not affect the amplitude of the AP, but increased the threshold by 13%. The half decay time was prolonged by 20% while the maximal rates of rise and fall were both reduced by 20% (Figure 4), reflecting a small inhibition of voltage-dependent sodium channels and decrease of the potassium conductance, respectively.

Effects on the endplate currents

Under control conditions, the amplitudes of e.p.cs recorded from the rat levator ani muscle fibres at membrane potentials ranging from -110 mV to $+50$ mV were linearly related to the clamped potentials. The time constant of e.p.c. ($\tau_{e.p.c.}$) increased exponentially as the membrane was hyperpolarized according to the relationship : $\tau\,(V) = \tau(0)\exp(-V/H)$, where $\tau(V)$ is the decay time constant at holding potential V, $\tau(0)$ is the decay time constant at 0 mV and H is a constant describing the voltage sensitivity of the decay phase (Magleby & Stevens, 1972). The reversal potential of the e.p.c. determined in these muscle fibres was -6.2 ± 1.1 mV ($n = 32$ fibres), similar to that obtained for the diaphragm muscle (Gibb & Marshall, 1984).

Incubation of Phen (10–100 μM) decreased the amplitudes of e.p.c. proportionately to the concentration producing nonlinearity of the current-voltage relationship at more negative membrane potentials (Figure 5). Within 30 min incubation of 10, 50 and 100 μM Phen, the mean e.p.c. amplitude was reduced to 33%, 43% and 8% at $+40$ mV; to 46%, 30% and 6% at -80 mV; and to 42%, 19% and 3% at -110 mV, of the respective control values (Figure 5, Table 1). At -80 mV holding potential, the e.p.c. rise time was not affected by low concentrations of Phen (10 and 50 μM), but it was reduced by 21% in the presence of 100 μM of the drug (Table 1). Phen (50 μM) also shortened the time constant of e.p.c. particularly at hyperpolarized membrane potentials (Figure 5). Thus at -80 mV and -110 mV holding potential, Phen (50 μM) reduced $\tau_{e.p.c.}$ to 55% and 33% of control values, respectively (Figure 5, Table 1), and decreased the voltage-dependence of $\tau_{e.p.c.}$ (H value: from -122 ± 6 mV, $n = 19$ to -285 ± 46 mV, $n = 17$). No further decrease of $\tau_{e.p.c.}$ was produced by the highest concentration of Phen (100 μM).

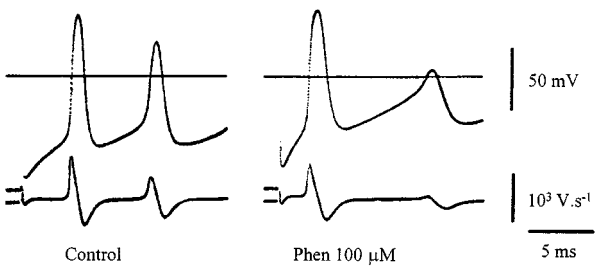

Figure 4 Directly elicited action potentials recorded at the extrajunctional region of surface fibres of glycerol-treated sartorius muscle of the frog, in control condition (left) and after 30 min exposure to phenthonium (Phen, 100 μM) (right). The horizontal line represent zero potential and lower trace is the first derivative (dv/dt) of the action potential.

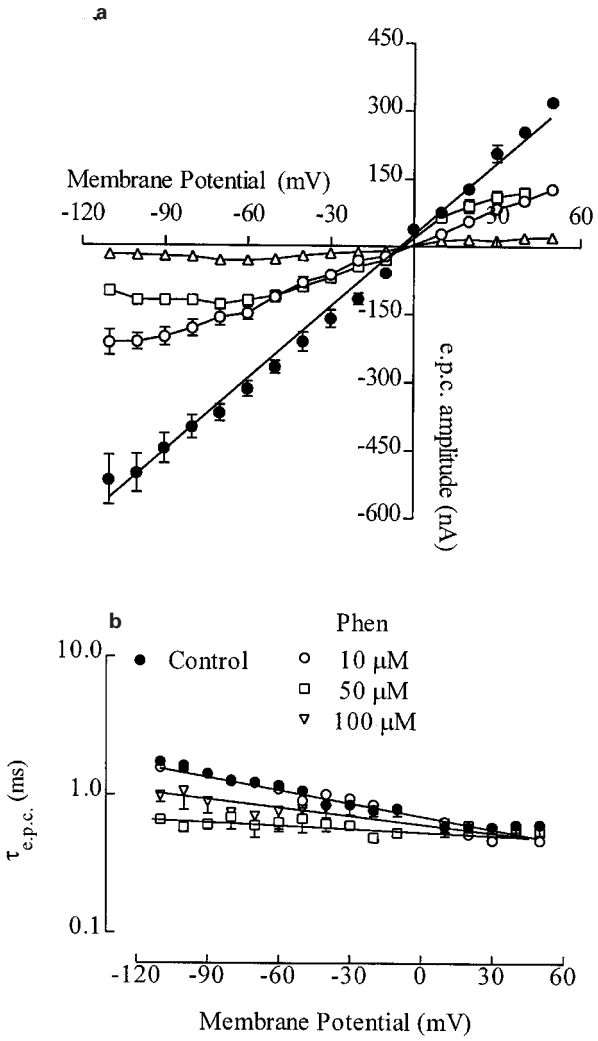

Figure 5 Current-voltage relationship of the e.p.c. peak amplitude (a) and semi-logarithmic plot of the time constant of e.p.c. decay ($\tau_{e.p.c.}$)(b) in control conditions and after 30 min exposure of the rat levator ani muscle to phenthonium 10, 50 and 100 μM. Symbols are means and vertical lines s.e. mean of 10 to 42 fibres in 4 to 8 muscles.

Table 1 Effect of phenthonium (Phen) on the peak amplitude, rise time and decay time constant ($\tau_{e.p.c.}$) of the endplate currents (e.p.cs) recorded in cut preparations of the rat levator ani muscle fibres at -80 mV (22–24°C)

Condition	*Rise time (ms)*	*Peak e.p.c. amplitude (nA)*	$\tau_{e.p.c.}$ *(ms)*
Control	0.63 ± 0.02	399.0 ± 25.0	1.25 ± 0.06
Phen 10 μM	0.67 ± 0.04	182.0 ± 18.0*	1.27 ± 0.05
Phen 50 μM	0.62 ± 0.01	121.0 ± 12.0*	0.69 ± 0.05*
Phen 100 μM	0.50 ± 0.01*	24.0 ± 0.4*	0.74 ± 0.08*

Results are means $\pm$ s.e.mean of 10 to 42 fibres in 4 to 8 muscles. *Different from control ($P < 0.05$).

Likewise no significant changes of $\tau_{e.p.c.}$ were detected at positive membrane potentials in the presence of either concentration of the drug (Figure 5).

Binding studies

Specific binding of [^{125}I]-BUTX (0.5 to 3 nM) to junctional ACh receptors of the innervated diaphragm muscle increased with time, stabilizing between 3 and 4 h of incubation. After 3 h incubation with 2 nM [^{125}I]-BUTX at 37°C, specific binding was 2.24 ± 0.20 fmol mg^{-1} tissue ($n = 12$) not different from that obtained with higher concentrations of the toxin. Previous incubation with Phen (0.1 – 100 μM) reduced binding of [^{125}I]-BUTX by 10 to 70% of control values (Figure 6). In parallel experiments TC (0.1 – 25 μM) reduced the toxin binding by 10 to 80% of control.

The relationship obtained from a plot of r-1 which reflects the degree of binding inhibition, against the concentrations of Phen and TC were parallel and had a slope not different from 1 (Figure 7), as predicted for a competitive antagonist (Colquhoun & Rang, 1976). The apparent K_I of Phen determined as the slope of the corresponding line was 24 μM, similar to the IC_{50} value (23 μM) obtained in the twitch experiments. Comparatively, the value of K_I estimated for TC in this analysis (0.7 μM) was of the same range reported by others (Colquhoun & Rang, 1976).

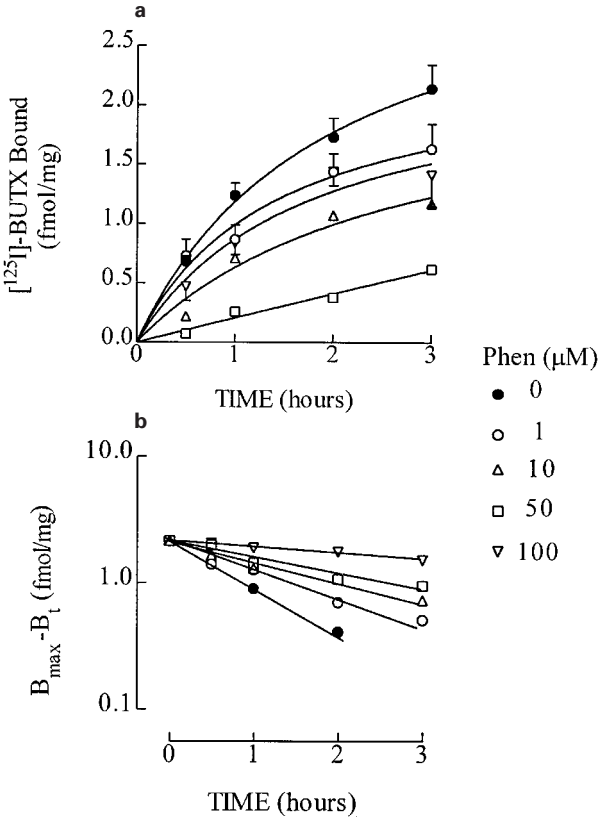

Figure 6 (a) Time-course of [^{125}I]-α-bungarotoxin ([^{125}I]-BUTX, 2 nM, 3 h, 37°C) specific binding to junctional ACh receptors of diaphragm muscle strips of the rat, in the absence and presence of phenthonium (Phen) at the indicated concentrations. Symbols and vertical lines are means and s.e. mean of 4 to 7 determinations. (b) Semi-logarithmic plots of the difference between specific maximal binding and specific binding at time t ($B_{max} - B_t$) against time, in the absence (0) and presence of Phen at the indicated concentrations. The rate constant (k) for toxin binding was determined from the slopes of the lines.

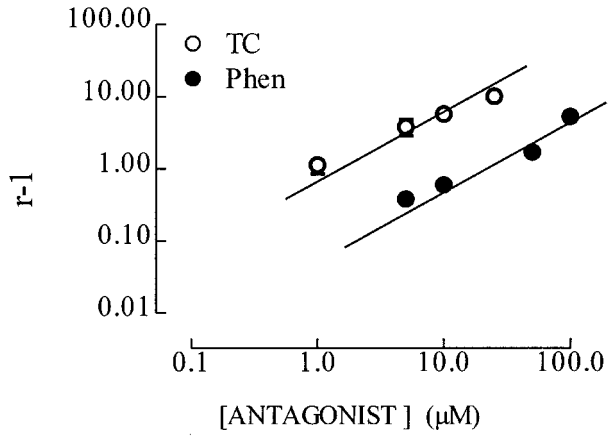

Figure 7 Inhibition of specific [^{125}I]-BUTX binding to junctional ACh receptors of diaphragm muscle strips by phenthonium (Phen) and (+)-tubocurarine (TC). The rate constants (k) for toxin binding determined from the slopes of the lines in Figure 6b were used to calculate the ratio of the rate constant for toxin binding in the absence of inhibitor to that in the presence of the inhibitor (r). The apparent dissociation constants (K_I) for Phen and TC were determined from the concentration at which $r - 1 = 1$.

Discussion

The data presented show that Phen, a quaternary derivative of (-)-hyoscyamine with antimuscarinic properties, blocked reversibly the indirectly-elicited muscle AP and the nerve-induced muscle twitches in both mammalian and amphibian muscles at concentrations previously shown to increase the spontaneous ACh release (Fann *et al*., 1990; Cysneiros *et al*., 1991; Souccar *et al*., 1994).

Phen also produced a small decrease in the rate of rise of the directly-evoked AP and prolonged its falling phase indicating that the drug slightly affects the voltage-dependent sodium and potassium channels. Similar effects on the muscle membrane excitability were also described for the muscarinic antagonist atropine (Lapa *et al*., 1974). However, these actions do not explain the neuromuscular blockade induced by Phen, because a prolonged decay of the muscle AP would be expected to potentiate rather than depress the muscle twitch.

Phen-induced depression of the nerve-elicited muscle twitch was accelerated in the presence of the anticholinesterase agent neostigmine, favouring a noncompetitive mechanism of neuromuscular blockade. As shown in the e.p.c. data, a low concentration of Phen (10 μM) produced a voltage-dependent decrease in the peak e.p.c. amplitude and induced non-linearity of the current-voltage relationship, indicating interaction of the drug with the ionic channel of the nicotinic receptor. Such effects were described for a diversity of pharmacological compounds known as noncompetitive blockers of the nicotinic ACh receptor (Barrantes *et al*., 1997), namely the quaternary local anaesthetic meproadifen (Maleque *et al*., 1982), the frog toxins histrionicotoxin (HTX) (Spivak *et al*., 1982) and gephyrotoxin (Souccar *et al*., 1984). High concentrations of Phen (50 and 100 μM) also caused shortening of the time constant of e.p.c. decay ($\tau_{e.p.c.}$) and a decrease of its voltage-dependence, a mechanism compatible with an open channel blockade (Spivak & Albuquerque, 1982). Shortening of $\tau_{e.p.c}$ was also described for other muscarinic antagonists like (−)-hyoscyamine (Albuquerque *et al*., 1980), atropine, scopolamine (Adler & Albuquerque, 1976; Adler *et al*., 1978; Feltz *et al*., 1977), and quinuclidynilbenzylate (QNB) (Schofield *et al*., 1981), the effect being attributed to binding of these drugs to

the activated ion channel. Like atropine (Adler & Albuquerque, 1976; Feltz *et al*., 1977) and QNB (Schofield *et al*., 1981), Phen did not affect the e.p.c. single exponential decay, suggesting similar kinetics with the ionic channel of all three muscarinic antagonists.

The more intense effect of Phen in decreasing the e.p.c. peak amplitude than shortening the e.p.c. decay indicated that the drug does not interact with the open channel conformation only. In fact, a high concentration of the drug (100 μM) also shortened the e.p.c. rise time, suggesting a probable interaction with the ionic channel before its activation by the agonist as reported for (−)-hyoscyamine (Albuquerque *et al*., 1980).

Both functional and binding data indicated that a receptor blockade action may contribute to the decrease in e.p.c. amplitude induced by Phen. Differently from that reported for atropine (Lapa *et al*., 1974) and HTX (Lapa *et al*., 1975), Phen protected the ACh receptor against irreversible α-bungarotoxin blockade, as indicated by recovery of the endplate potentials in the mouse diaphragm preparations. However, in similar experiments, the standard competitive antagonist of the nicotinic ACh receptor, tubocurarine (TC, 3 μM) at a concentration equieffective to that of Phen (100 μM) did not protect the receptor against α-bungarotoxin blockade. Protection by TC was obtained only in the presence of concentrations higher than the IC_{50} value. On the other hand, both Phen and tubocurarine retarded [^{125}I]-α-bungarotoxin binding to junctional ACh receptors of the rat diaphragm, TC being the more effective. The linear relationship between the degree of binding inhibition expressed as (r-1) and the concentration of Phen and TC, as well as the parallelism between the inhibition lines, were indications of similar competitive mechanisms (Colquhoun & Rang, 1976), albeit with different affinities.

Noncompetitive blockers are known to bind to different allosteric ACh binding sites of the nicotinic receptor. According to Changeux and coworkers, the allosteric binding sites for these antagonists may occur at either a unique high-affinity site sensitive to HTX, located within the ion channel of the receptor molecule, or to a diversity of low-affinity sites insensitive to HTX, possibly located at the interface of the receptor with membrane lipids (Heidman *et al*., 1983; Galzi *et al*., 1991). Based on these studies, our functional, electrophysiological and binding data suggest that Phen exhibits a unique action blocking the neuromuscular transmission through interaction with a site(s) that affects both ACh binding to its recognition site and ionic channel conductance, in contrast to the reported for other antimuscarinic compounds (Albuquerque *et al*., 1980).

The results obtained in the receptor protection against BUTX and those obtained in binding studies contrast when the IC_{50} values of Phen and TC are compared. These observations indicate that in functional studies competition does not solely occur between Phen and BUTX at the ACh binding site. On the other hand, an interaction of both Phen and TC with different sites (ACh recognition site and ionic channel) at the nicotinic receptor does not support the difference observed between functional and binding data. In fact, Phen was shown to increase the spontaneous ACh release (Fann *et al*., 1990; Cysneiros *et al*., 1991; Souccar *et al*., 1994) which may also contribute to the protection against α-toxin binding. It is noteworthy that, at concentrations equieffective to those of Phen on the blockade of neuromuscular transmission, TC did not affect the m.e.p.p. frequency (Fann *et al*., 1990) and did not protect against the toxin binding.

In summary, the data presented show that the muscarinic antagonist Phen exerts multiple postjunctional actions, blocking noncompetitively and reversibly the neuromuscular transmission in both mammalian and amphibian muscles. The neuromuscular blockade was related to the interaction of the drug with a site(s) that affects both the agonist recognition binding and ionic channel conductance of the ACh receptor. In contrast to muscarinic antagonists, Phen protected the ACh receptor against α-bungarotoxin blockade, an effect that may also be related to its previously reported prejunctional effect, increasing spontaneous ACh release.

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References

ADLER, M. & ALBUQUERQUE, E.X. (1976). An analysis of the action of atropine and scopolamine on the end-plate current of frog sartorius muscle. *J. Pharmacol. Exp. Ther.,* **196,** 360 – 372.

ADLER, M., ALBUQUERQUE, E.X. & LEBEDA, F.J. (1978). Kinetic analysis of end plate currents altered by atropine and scopolamine. *Mol. Pharmacol.,* **14,** 514 – 529.

ALBUQUERQUE, E.X., ADLER, M., SPIVAK, C.E. & AGUAYO, L. (1980). Mechanism of nicotinic channel activation and blockade. *Ann. New York Acad. Sci.,* **358,** 204 – 328.

BARRANTES, G.E., ORTELLS, M.O. & BARRANTES, F.J. (1997). Screening structural-functional relationships of neuropharmacologically active organic compounds at the nicotinic acetylcholine receptor. *Neuropharmacology,* **36,** 269 – 279.

BOWMAN, W.C., PRIOR, C. & MARSHALL, I.J. (1990). Presynaptic receptors in the neuromuscular junction. *Ann. New York Acad. Sci.,* **604,** 548 – 557.

COLQUHOUN, D. & RANG, H.P. (1976). Effects of inhibitors on the binding of iodinated α-bungarotoxin to acetylcholine receptors in rat muscle. *Mol. Pharmacol.,* **12,** 529 – 535.

CYSNEIROS, R.M., LIMA-LANDMAN, M.T., SOUCCAR, C. & LAPA, A.J. (1991). Phenthonium induces a transient increase of acetylcholine efflux from motor nerve terminals. *Braz. J. Med. Biol. Res.,* **24,** 1055 – 1058.

FANN, M.L., SOUCCAR, C. & LAPA, A.J. (1990). Phenthonium, a quaternary derivative of (−)-hyoscyamine, enhances the spontaneous release of acetylcholine at rat motor nerve terminals. *Br. J. Pharmacol.,* **100,** 441 – 446.

FELTZ, A., LARGE, W.A. & TRAUTMANN, A. (1977). Analysis of atropine action at the frog neuromuscular junction. *J. Physiol.,* **269,** 109 – 130.

GAGE, P.W. & EISENBERG, R.S. (1969). Action potentials, after potentials, and excitation contraction coupling in frog sartorius fibers without transverse tubules. *J. Gen. Physiol.,* **53,** 289 – 310.

GALZI, J.-L., REVAH, F., BESSIS, A. & CHANGEUX, J.-P. (1991). Functional architecture of the nicotinic acetylcholine receptor: from electric organ to brain. *Annu. Rev. Pharmacol.,* **31,** 37 – 72.

GIBB, A.J. & MARSHALL, I.G. (1984). Pre and post-junctional effects of tubocurarine and other nicotinic antagonists during repetitive stimulation in the rat. *J. Physiol.,* **351,** 275 – 297.

GLAVINOVIC, M.I. (1979). Presynaptic action of curare. *J. Physiol.,* **290,** 499 – 506.

HEIDMAN, T., OSWALD, R.E. & CHANGEUX, J.-P. (1983). Multiple sites of action for noncompetitive blockers on acetylcholine receptor rich membrane fragments from Torpedo marmorata. *Biochemistry,* **22,** 3112 – 3127.

LAPA, A.J., ALBUQUERQUE, E.X. & DALY, J. (1974). An electrophysiological study of the effects of d-tubocurarine, atropine and α-bungarotoxin on the cholinergic receptor in innervated and chronically denervated mammalian skeletal muscles. *Exp. Neurol.,* **43,** 375 – 398.

LAPA, A.J., ALBUQUERQUE, E.X., SARVEY, J.M., DALY, J. & WITKOP, B. (1975). Effects of histrionicotoxin on the chemosensitive and electrical properties of skeletal muscle. *Exp. Neurol.,* **47,** 558 – 580.

MAGLEBY, K. & STEVENS, C.F. (1972). The effect of voltage on the time course of endplate currents. *J. Physiol.,* **223,** 151 – 172.

MALEQUE, M.A., SOUCCAR, C., COHEN, J.B. & ALBUQUERQUE, E.X. (1982). Meproadifen reaction with the ionic channel of the acetylcholine receptor: potentiation of agonist-induced desensitization at the frog neuromuscular junction. *Molec. Pharmacol.,* **22,** 636 – 647.

SCHILD, O. (1947). pA_2, a new scale for the measurement of drug antagonism. *Br. J. Pharmacol. Chemother.,* **2,** 189 – 206.

SCHOFIELD, G.C., WARNICK, J.E. & ALBUQUERQUE, E.X. (1981). Elucidation of the mechanism and site of action of quinuclydinyl benzylate (QNB) on the electrical excitability and chemosensitivity of the frog sartorius muscle. *Cell. Molec. Neurobiol.,* **1,** 209 – 230.

SMITH, D.O. & CHAPMAN, M.R. (1987). Acetylcholine receptor binding properties at the rat neuromuscular junction during aging. *J. Neurochem.,* **48,** 1834 – 1841.

SOUCCAR, C., LAPA, A.J. & VALLE, J.R. (1982). The influence of testosterone on neuromuscular transmission of hormone sensitive mammalian skeletal muscles. *Muscle Nerve,* **5,** 232 – 237.

SOUCCAR, C., SOLDERA, J.C., CYSNEIROS, R.M., GONÇALO, M.C. & LAPA, A.J. (1994). Prejunctional effect of quaternary derivatives of l-hyoscyamine at the rat neuromuscular junction. A structure-activity relationship study. *Gen. Pharmacol.* **25,** 1397 – 1404.

SOUCCAR, C., VARANDA, W.A., DALY, J.W. & ALBUQUERQUE, E.X. (1984). Interactions of gephyrotoxin with the acetylcholine receptor ionic channel complex:I-Blockade of the ionic channel. *Molec. Pharmacol.,* **25,** 384 – 394.

SOUCCAR, C., YAMAMOTO, L.A., GONÇALO, M.C. & LAPA, A.J. (1991). Androgen regulation of the nicotinic acetylcholine receptor-ionic channel in a hormone-dependent skeletal muscle. *Braz. J. Med. Biol. Res.,* **24,** 1051 – 1054.

SPIVAK, C.E. & ALBUQUERQUE, E.X. (1982). Dynamic properties of the nicotinic acetylcholine receptor ionic channel complex: activation and blockade. In *Progress in Cholinergic Biology: Models of Cholinergic Synapses*, ed. Hanin, I. & Goldberg, A.M. pp. 323 – 357. New York: Raven Press.

SPIVAK, C.E., MALEQUE, M.A., OLIVEIRA, A.C., MASUKAWA, L.M., TOKUYAMA, T., DALY, J.W. & ALBUQUERQUE, E.X. (1982). Actions of the histrionicotoxins at the ion channel of the nicotinic acetylcholine receptor and at the voltage-sensitive ion channels of muscle membranes. *Mol. Pharmacol.,* **21,**351 – 361.

TAKEUCHI, A. & TAKEUCHI, N. (1959). Active phase of frog's endplate potential. *J. Neurophysiol.,* **22,** 395 – 411.

WESSLER, I. (1989). Control of transmitter release from the motor nerve by presynaptic nicotinic and muscarinic autoreceptors. *Trends Pharmacol. Sci.,* **10,** 110 – 114.

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