Phenthonium, a quaternary derivative of (–)-hyoscyamine, enhances the spontaneous release of acetylcholine at rat motor nerve terminals

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- 1 A quaternary derivative of (–)-hyoscyamine, phenthonium (Phen) induced a concentration-dependent increase in the rate of spontaneous quantal release of acetylcholine (ACh) at the mammalian neuromuscular junction, as shown by intracellular recordings of the miniature endplate potentials (m.e.p.ps) in rat diaphragm muscles.
- 2 The prejunctional effect of Phen (10–50 μM) was reversible, unrelated to temperature (22°–35°C), unaltered by either changes in $[\text{Ca}^{2+}]_o$ or by high $[\text{Mg}^{2+}]_o$, and was not induced by membrane depolarization.
- 3 Simultaneously, Phen reduced the amplitude of m.e.p.ps by a postjunctional action.
- 4 The muscarinic agonist oxotremorine did not prevent the increase in m.e.p.p. frequency induced by Phen. Cholinesterase inhibition with neostigmine potentiated the prejunctional effect induced by a low (20 μM) but not a high (50 μM) concentration of Phen.
- 5 The increase in m.e.p.p. frequency induced by Phen was not influenced by previous incubation with either atropine (0.01–10 μM) or (+)-tubocurarine (0.05–0.1 μM). Each antagonist however, intensified the postjunctional effect of Phen.
- 6 Phen (20 μM) did not influence the quantal contents of e.p.ps in cut-muscle preparations or in the presence of high $[\text{Mg}^{2+}]_o$. A high concentration of Phen (50 μM) increased the rundown of e.p.p. trains evoked at 10–50 Hz.
- 7 The results indicate that the facilitatory prejunctional action of Phen cannot be explained by an antimuscarinic activity. A possible interaction of the antagonist with putative prejunctional nicotinic cholinergic receptors however, was not excluded.

Introduction

The existence of prejunctional acetylcholine (ACh) receptors which modulate the transmitter release at motor nerve terminals has been suggested frequently (Miyamoto, 1978; Kilbinger, 1984; Bowman *et al.*, 1984; 1988). The type of cholinergic sites involved in the feedback regulation however, is controversial. Muscarinic inhibitory (Duncan & Publicover, 1979; Michaelson *et al.*, 1979; Abbs & Joseph, 1981; Somogyi *et al.*, 1987) and excitatory (Das *et al.*, 1978; Wali *et al.*, 1987; Wessler *et al.*, 1987) prejunctional cholinergic receptors have been reported at vertebrate neuromuscular junctions and in the *Torpedo* electric organ. In the presence of tubocurarine, a frequency-dependent decrease of evoked ACh release was obtained which suggested the presence of nicotinic prejunctional cholinergic receptors mediating a positive feedback control of evoked transmitter release (Glavinovic, 1979a; Magleby *et al.*, 1981; Gibb & Marshall, 1984; Wessler *et al.*, 1986; Matzner *et al.*, 1988). Evidence has also been presented against both muscarinic (Gundersen & Jenden, 1980; Haggblad & Heilborn, 1983) and nicotinic (Beranek & Vyskocil, 1967; Auerbach & Betz, 1971; Jones & Salpeter, 1983) prejunctional cholinergic regulation at the neuromuscular junction.

Most of the data supporting a prejunctional cholinergic modulation have been provided by experiments on the nerve evoked ACh release largely performed in the presence of either high K^+ or anticholinesterase agents. These agents could have accounted for the conflicting results (Kilbinger, 1984; Somogyi *et al.*, 1987). Few studies have presented evidence for a pre-

junctional cholinergic regulation of spontaneous transmitter release. In the rat diaphragm muscle, carbachol increased the frequency of miniature endplate potentials (m.e.p.ps), an effect which was blocked by tubocurarine, indicating the existence of prejunctional nicotinic receptors (Miyamoto & Volle, 1974). At frog neuromuscular junctions however, an increase in the m.e.p.p. frequency was detected in the presence of atropine, suggesting the involvement of prejunctional muscarinic modulation (Duncan & Publicover, 1979).

The present study investigated the prejunctional effects of a quaternary derivative of (–)-hyoscyamine, phenthonium (Figure 1), on transmitter release at the mammalian neuromuscular junction. This drug has antimuscarinic but no ganglionic blocking activity (Della Bella *et al.*, 1968; Teotino *et al.*, 1968), and is a noncompetitive antagonist of neuromuscular transmission (Souccar *et al.*, 1986). The results showed that phenthonium increased the spontaneous but not

Figure 1 Chemical structure of phenthonium.

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the evoked ACh release, suggesting a prejunctional cholinergic modulation of the resting quantal transmitter release.

Methods

Phrenic nerve-diaphragm muscle preparations from female Wistar rats (200–250 g) were used. The left hemidiaphragm was pinned down, slightly stretched on Sylgard (Dow Corning), placed in a 10 ml muscle chamber and continuously perfused with physiological solution. The resting membrane potentials (RMP), miniature endplate potentials (m.e.p.ps) and endplate potentials (e.p.ps) with rise time of 1 ms or less, were recorded intracellularly at endplate regions, at room temperature (22–25°C). Glass microelectrodes containing 3 M KCl (resistance 6–10 MΩ) and a high impedance unity gain electrometer (WP Instruments 750) were employed. A silver/silver chloride electrode was used as reference. When necessary, the temperature of the bathing solution was raised by circulating heated water through a chamber beneath the bath floor.

The normal physiological solution consisted of (mM): NaCl 135, KCl 2.5, MgCl₂ 1, CaCl₂ 2, NaHCO₃ 15, NaH₂PO₄ 1 and glucose 11, gassed with 95% CO₂ and 5% O₂ (pH 7.3–7.4). For e.p.p. recording, muscle contractions were prevented by either raising the Mg²⁺ concentration to 15 mM in normal physiological solution (Ca²⁺ = 2 mM) or by cutting the muscle fibres across their length approximately 2 mm at each extremity (Barstad & Lilleheil, 1968). Osmolarity of the saline solution was maintained by correction of the NaCl concentration.

E.p.ps were evoked by stimulating the phrenic nerve via a bipolar platinum electrode at 0.5 Hz, 0.05 ms and supra-maximal voltage. Trains of 25 e.p.ps were elicited by repetitive stimulation (25, 50 and 100 Hz) at intervals of 60–90 s. For quantal content determination, at least 100 e.p.ps were recorded at each endplate, photographed from the oscilloscope and measured on film under magnification. M.e.p.ps were simultaneously recorded on a Mingograph chart recorder (Elema-Schonander, Sweden) and measured. The amplitudes of e.p.ps were corrected for non-linear summation (MacLachlan & Martin, 1981) assuming the reversal potential for ACh in the diaphragm muscle fibres as 0 mV (Alemá *et al.*, 1981). The amplitudes of m.e.p.ps from the cut-muscle fibres were corrected to a mean RMP of –45 mV determined experimentally, using the relationship: $\text{m.e.p.p.}_c = \text{m.e.p.p.} \times 45/\text{RMP}$, where m.e.p.p._c and m.e.p.p. are the corrected and recorded m.e.p.p. amplitude, respectively (Thomsen & Wilson, 1983). The quantal content of the e.p.ps (m) was calculated by the direct method as the ratio of mean e.p.p. amplitude to the mean m.e.p.p. amplitude (Hubbard *et al.*, 1969).

Materials

Drugs used were: atropine sulphate, oxotremorine sesquifumarate, neostigmine bromide, (+)-tubocurarine hydrochloride and tetrodotoxin (Sigma). Phentonium [N-(4-phenyl)-phenacyl (–)-hyoscyamine] bromide was kindly provided by Zambon Laboratories (Italy). Stock solutions of all drugs were prepared in de-ionized water, and phentonium was dissolved in 0.01 N hydrochloric acid (12%). All muscle preparations were allowed to equilibrate for 30 min in the normal or modified physiological solution before a control period of recording. Incubation with drugs was done by exchanging the bathing medium with a physiological solution containing appropriate concentrations of the drugs being tested.

Statistics

Normally distributed data were expressed as means $\pm$ s.e.mean, and differences among groups were determined by

Student's *t* test. Data of non-normal distributions (m.e.p.p. frequency) were presented as geometric means $\pm$ approximate s.e.; the Mann-Whitney test was used for comparison of data (Colquhoun, 1971). Differences among data were considered significant at $P < 0.05$.

Results

Spontaneous transmitter release

In normal physiological solution, the mean RMP at room temperature (22–25°C) was -81 ± 1 mV; the frequency and amplitude of m.e.p.ps were respectively, 1.20 ± 0.15 s⁻¹ and 0.76 ± 0.02 mV (139 fibres of 12 muscles). Phentonium (Phen, 10–50 μM) did not alter the RMP, but induced a concentration-dependent increase in m.e.p.p. frequency, after 10 min. The effect was maximal within 25–30 min of incubation, being 1.99 ± 0.34 s⁻¹, 2.68 ± 0.37 s⁻¹, and 6.46 ± 0.70 s⁻¹ in the presence of 10, 20 and 50 μM Phen, respectively (Figure 2). Simultaneously, the amplitude of m.e.p.ps was decreased by 30–35% of control in the presence of low concentrations of Phen (10 and 20 μM). Higher concentrations of Phen (50–100 μM) caused intense postjunctional blockade, leading to an apparent decrease of the m.e.p.p. frequency in few cells. After 5 min washout of the drug however, the postjunctional blockade was reversed and m.e.p.p. frequencies higher than 6 s⁻¹ were recorded. To assess the prejunctional actions of Phen underlying the increase of m.e.p.p. frequency, a concentration of the antagonist that induced a small postjunctional blockade (20 μM) was employed in most of the following experiments.

Effect of temperature Raising the temperature of the bathing medium from 22°C to 30°C did not affect the frequency of m.e.p.ps (1.11 ± 0.24 s⁻¹, 43 fibres/4 muscles), but at 35°C frequency was increased 2.8 fold (3.40 ± 0.34 s⁻¹, 45 fibres/4 muscles). Incubation with Phen (20 μM) at both temperatures increased the frequency of m.e.p.ps to 246% and 188% of the respective control at 30°C and 35°C.

Figure 2 Time course of the effects of phentonium (Phen; 10 μM ○; 20 μM △ and 50 μM ◇) on the frequency of miniature endplate potentials (m.e.p.ps) recorded from rat diaphragm muscle fibres at 22–25°C. Phen was incubated at time zero, and m.e.p.p. frequency was determined at 5 min intervals. Symbols and vertical bars are geometric means $\pm$ approximate s.e. of 20–60 fibres in 4 to 6 muscles. Inset: typical m.e.p.p. recordings alone and after 30 min exposure to 20 μM Phen. Calibration bars are 10 mV (control) or 5 mV (Phen) and 50 ms.

The amplitude of m.e.p.s was not altered by raising the temperature either to 30°C (0.72 ± 0.03 mV) or to 35°C (0.70 ± 0.01 mV), and was reduced in presence of Phen ($20 \mu\text{M}$) by 33% of control at both temperatures. The RMP values recorded in the absence or presence of Phen ($20 \mu\text{M}$) were not altered by changes of temperature.

Effect of extracellular Ca^{2+} Reducing $[\text{Ca}^{2+}]_o$ in the physiological solution to 0.5 and 1.0 mM decreased m.e.p.p. frequency to respectively, $0.61 \pm 0.27 \text{ s}^{-1}$ (42 fibres/4 muscles) and $0.77 \pm 0.29 \text{ s}^{-1}$ (46 fibres/4 muscles), whereas raising the $[\text{Ca}^{2+}]_o$ to 5 mM increased the m.e.p.p. frequency to $2.41 \pm 0.43 \text{ s}^{-1}$ (39 fibres/3 muscles). Incubation with Phen ($20 \mu\text{M}$) for 30 min did not alter the frequency of m.e.p.s in a medium containing 0.5 mM $[\text{Ca}^{2+}]_o$, but increased that recorded in presence of 1 mM $[\text{Ca}^{2+}]_o$ to 197% of control. In the presence of 5 mM $[\text{Ca}^{2+}]_o$, the effect of Phen ($20 \mu\text{M}$) did not differ from that obtained in normal $[\text{Ca}^{2+}]_o$ solution, raising the frequency of m.e.p.s to 210% of control after 10 min. The relationship of the log m.e.p.p. frequency against the log $[\text{Ca}^{2+}]_o$ determined in the absence and presence of Phen was linear, with slope values of 0.71 and 0.74, respectively. The curve in the presence of Phen showed a parallel shift to the left of that obtained in its absence (Figure 3).

Raising the Mg^{2+} concentration (15 mM) in normal $[\text{Ca}^{2+}]_o$ (2 mM) solution did not alter the control frequency of m.e.p.s ($1.11 \pm 0.39 \text{ s}^{-1}$, 32 fibres/3 muscles), nor did it affect the increase in m.e.p.p. frequency induced by $20 \mu\text{M}$ Phen (230% of control).

The amplitude of m.e.p.s recorded in the presence of various $[\text{Ca}^{2+}]_o$ (0.5 mM: 0.78 ± 0.03 mV; 1 mM: 0.87 ± 0.06 mV; 5 mM: 0.82 ± 0.04 mV, $n = 114$ to 252 m.e.p.s) did not differ from control, but it was reduced by 35% to 50% after 30 min exposure to $20 \mu\text{M}$ Phen.

No significant changes in RMP values were detected over the range of $[\text{Ca}^{2+}]_o$ tested in either controls or after incubation with Phen.

Effect of neostigmine Neostigmine ($0.5 \mu\text{M}$) caused spontaneous twitches of the muscle fibres which were prevented by tetrodotoxin (TTX, $0.5 \mu\text{M}$). TTX (0.5 – $1.0 \mu\text{M}$) alone did not

Figure 3 Effect of increasing the external concentration of Ca^{2+} (abscissa scale), on the frequency of miniature endplate potentials (m.e.p.s, ordinate scale) of rat diaphragm muscle fibres recorded at 22°–25°C, in control preparations (○) and following 25–30 min incubation with phenthonium $20 \mu\text{M}$ (●). Symbols and vertical bars are geometric means $\pm$ approximate s.e. of 15–40 fibres in 4 muscles.

affect the ability of 20 and $50 \mu\text{M}$ Phen to increase m.e.p.p. frequency to respectively, 240% and 528% of control ($1.24 \pm 0.27 \text{ s}^{-1}$; 90 fibres/4 muscles). Neostigmine ($0.5 \mu\text{M}$) did not alter the mean frequency of m.e.p.s after 30 min ($1.22 \pm 0.17 \text{ s}^{-1}$; 129 fibres/8 muscles), although few cells presented m.e.p.p. frequencies up to 10 s^{-1} . Phen ($20 \mu\text{M}$) added to muscles preincubated with neostigmine ($0.5 \mu\text{M}$) increased the m.e.p.p. frequency to $4.06 \pm 0.52 \text{ s}^{-1}$ (19 fibres/7 muscles) after 15 min (Figure 4). The results were 1.5 fold higher than those obtained in presence of Phen ($20 \mu\text{M}$) alone. A higher concentration of Phen ($50 \mu\text{M}$) added after preincubation with neostigmine ($0.5 \mu\text{M}$) increased the m.e.p.p. frequency to the same levels as those found in the presence of Phen ($50 \mu\text{M}$) alone (523% of control).

The mean amplitude of m.e.p.s determined after 30 min incubation with neostigmine ($0.5 \mu\text{M}$) was increased by 47% of control (0.70 ± 0.04 mV, $n = 210$ m.e.p.s). Those values were not altered after 30 min exposure to neostigmine ($0.5 \mu\text{M}$) plus Phen ($20 \mu\text{M}$). On the other hand, neostigmine ($0.5 \mu\text{M}$) together with Phen ($50 \mu\text{M}$) reduced m.e.p.p. amplitude to those values obtained with Phen alone (20% of control).

Effect of oxotremorine The mean values of RMP (-81 ± 2 mV) and m.e.p.p. frequency ($1.21 \pm 0.18 \text{ s}^{-1}$; 90 fibres/6 muscles) were not altered by $1 \mu\text{M}$ oxotremorine, although m.e.p.p. amplitude was decreased by 20% of control (0.65 ± 0.08 mV). Higher concentrations of oxotremorine (5 and $10 \mu\text{M}$) each depolarized the muscle fibres by some 20 mV causing spontaneous twitching. The frequency of m.e.p.s however, did not differ from control over 1 h.

Previous incubation with oxotremorine ($1 \mu\text{M}$) for 30 min did not prevent the increase in m.e.p.p. frequency induced by Phen ($20 \mu\text{M}$), which reached 213% of the values determined in presence of oxotremorine alone ($1.0 \pm 0.31 \text{ s}^{-1}$, 52 fibres/4 muscles). These values did not differ from those obtained with equal concentration of Phen alone (Figure 4). The post-junctional effect of Phen ($20 \mu\text{M}$) however, was potentiated by oxotremorine ($1 \mu\text{M}$) reducing the m.e.p.p. amplitude by some 80% of control.

Effect of atropine and (+)-tubocurarine Exposure to atropine (0.01 – $10 \mu\text{M}$) and (+)-tubocurarine (Tc, 0.05 – $0.1 \mu\text{M}$) each for 30 min did not alter RMP or m.e.p.p. frequency; the amplitude of m.e.p.s was reduced by 20–30% and 50–70%, respectively.

Figure 4 Time course of effects of phenthonium (Phen, $20 \mu\text{M}$) on the frequency of miniature endplate potentials (m.e.p.s in s^{-1}) recorded from rat diaphragm muscle fibres, in presence of Phen alone (●), and after additional preincubation with neostigmine (Neo, $0.5 \mu\text{M}$, ○) or oxotremorine (Oxo, $1 \mu\text{M}$, △). Incubation began at the time indicated by arrows. Control values (C) were obtained from 106 fibres/9 muscles for neostigmine, and 89 fibres/7 muscles for oxotremorine. Symbols and vertical bars are geometric means $\pm$ approximate s.e. of 15–38 fibres in 5–11 muscles.

Table 1 Effect of phentonium on the quantal content of endplate potentials (m) determined in 'cut' diaphragm muscle fibres and in intact muscles bathed in high $[Mg^{2+}]_o$ solution

Condition	e.p.p. amplitude (mV)	m.e.p.p. amplitude (mV)	m
'Cut-muscle'			
Control	17.89 $\pm$ 1.97* (17/6)	0.64 $\pm$ 0.08	31.0 $\pm$ 3.9
Phentonium (20 μ M)	11.55 $\pm$ 1.71* (17/6)	0.52 $\pm$ 0.08	25.3 $\pm$ 3.8
15 mM Mg^{2+} solution			
Control	1.74 $\pm$ 0.36 (16/5)	0.37 $\pm$ 0.03	4.69 $\pm$ 0.80
Phentonium (20 μ M)	1.61 $\pm$ 0.23 (17/4)	0.38 $\pm$ 0.02	4.31 $\pm$ 0.61

* Values are means $\pm$ s.e.means with the number of fibres/number of muscle preparations in parentheses.

* Significantly different from control ($P < 0.05$).

Figure 5 Decay of endplate potentials (e.p.ps) elicited at different frequencies of stimulation in cut diaphragm muscles of the rat, in the absence (○) and presence of phentonium (20 μ M, Δ ; 50 μ M, $\diamond$). The nerve was stimulated by trains of 25 pulses each at the indicated frequency of stimulation (abscissa scale), and the e.p.p. decay was expressed as a % of the first e.p.p. amplitude (ordinate scale). Symbols are means $\pm$ s.d. of 6–12 fibres in 4 muscles. **Figure 6** Time course of the decay of endplate potentials (e.p.ps) evoked by nerve stimulation, at 25 Hz, of cut diaphragm muscle fibres of the rat, obtained in the absence (●) and presence of phentonium (20 μ M – Δ ; 50 μ M – $\diamond$) or tubocurarine (0.5 μ M – ○). The decay of e.p.ps was expressed as a % of the first e.p.p. amplitude (ordinate). Symbols are means of 7–12 fibres in 4 muscles; s.d. at each point ranged from 3 to 10% and was omitted for clarity.

Addition of Phen (20 μ M) to muscles preincubated with atropine (10 μ M) or Tc (0.1 μ M) increased the frequency of m.e.p.ps to respectively, $2.17 \pm 0.39 s^{-1}$ (17 fibres/3 muscles) and $2.31 \pm 0.57 s^{-1}$ (16 fibres/3 muscles) after 30 min. Those results did not differ from those obtained in the presence of Phen (20 μ M) alone. The amplitude of m.e.p.ps was further reduced by adding Phen and atropine (40%) or Tc (80%). Phen (20 μ M) together with higher concentrations of atropine (1 μ M) or Tc (1 μ M) reduced the amplitudes of m.e.p.ps to values equal to or below 0.1 mV, making the recordings unreliable.

Evoked transmitter release

To investigate the effect of Phen on the evoked transmitter release, the quantal content of e.p.ps (m) were determined in cut-muscle preparations and in intact muscles bathed in high $[Mg^{2+}]_o$ containing physiological solution. The RMP recorded from 'cut' diaphragm muscle fibres was -43 ± 1 mV (39 fibres/16 muscles), and was not altered after 30 min incubation with Phen (20 μ M). The mean values of m and m.e.p.p. amplitude did not differ from controls, although the e.p.p. amplitude was reduced by some 35% (Table 1). In intact muscle fibres bathed in a high $[Mg^{2+}]_o$ medium, Phen (20 μ M) did not change e.p.p. or m.e.p.p. amplitude, or m values from the respective controls (Table 1).

The effect of Phen was also examined on the decay of e.p.p. amplitudes evoked by repetitive nerve stimulation. Under control conditions, the amplitude of trains of e.p.ps induced at 10, 25 and 50 Hz in the cut rat diaphragm was initially increased transiently then decreased to a plateau level. The amplitude of the last (25th) e.p.p. expressed as % of the first e.p.p. reached $75.0 \pm 7.0\%$; $73.1 \pm 10.2\%$ and $62.0 \pm 10.1\%$ (means $\pm$ s.d.) at respectively, 10, 25 and 50 Hz. After 30 min incubation with Phen (20 and 50 μ M) the amplitude of the first e.p.p. was reduced by 40 and 46% of control values (10.03 ± 1.49 mV). Incubation with Phen (20 μ M) for 30 min did not alter the decay of e.p.p. trains recorded at 10–50 Hz. However, a high concentration of Phen (50 μ M) caused a faster decline of the e.p.p. trains, reaching the amplitude of the 25th e.p.p. $64.8 \pm 5.8\%$; $60.8 \pm 4.6\%$ and $47.0 \pm 4.7\%$ of the first e.p.p. at 10, 25 and 50 Hz, respectively (Figure 5). In similar experiments, Tc (0.5 μ M) induced a more intense e.p.p. rundown, reducing the amplitude of the 25th e.p.p. to $47.8 \pm 5.2\%$ and $49.5 \pm 3.1\%$ at 10 and 25 Hz respectively (Figure 6).

Discussion

A quaternary derivative of (–)-hyoscyamine, phentonium (Phen), induced a concentration-dependent increase in the rate of spontaneous quantal transmitter release but did not affect the RMP in the rat neuromuscular junction. This prejunctional effect was reversible thus eliminating the physical disruption of the nerve terminal membrane or intraterminal organelles and persisted in the presence of tetrodotoxin, excluding an activation of the voltage sensitive Na^+ channels at the nerve terminal membrane as a mechanism of action of the drug.

Raising the temperature in control muscles increased m.e.p.p. frequency (Hubbard *et al.*, 1969), without altering the prejunctional effect of Phen. In fact, at 35°C Phen induced a smaller increase of m.e.p.p. frequency than that obtained at 22°C or 30°C, suggesting a saturation level for the facilitatory effect of Phen. This limitation was not observed after raising the $[Ca^{2+}]_o$.

Increase in the free intraterminal Ca^{2+} concentration is known to raise the m.e.p.p. frequency (Katz & Miledi, 1969; Cohen *et al.*, 1974). The intraterminal Ca^{2+} concentration in its turn depends on the ion influx and Ca^{2+} regulating systems of intracellular organelles (Blaustein *et al.*, 1978; 1980). Changes of extracellular Ca^{2+} concentration carried

out in the presence of Phen caused a proportional increase of the m.e.p.p. frequency. As shown in Figure 3, the relationship of the log of m.e.p.p. frequency against the log of Ca^{2+} concentration obtained in the presence of Phen was shifted to the left of that obtained in control muscles, without alteration of the slope. These results indicate that the agent enhances the physiological mechanism of calcium-dependent quantal transmitter release. Although the effect was abolished in presence of low extracellular Ca^{2+} concentration (0.5 mM), it was still obtained in high- Mg^{2+} solution, indicating that Phen does not increase the spontaneous acetylcholine (ACh) release by promoting the influx of Ca^{2+} in the nerve terminal. A direct stimulating action on Ca^{2+} release from its internal stores also seems unlikely, since the compound is a quaternary derivative and therefore, hardly penetrates the nerve terminal. Nevertheless, one cannot eliminate a possible disruption of the intraterminal Ca^{2+} buffering systems, indirectly triggered through interaction of Phen with presynaptic receptors on the external surface of the nerve terminal membrane, resulting in hastened exocytosis.

In view of the cholinolytic actions of Phen, the increase in m.e.p.p. frequency induced by the antagonist may arise from an interaction with presynaptic cholinergic receptors, either muscarinic or nicotinic. In our experimental conditions, the muscarinic agonist oxotremorine did not influence the spontaneous ACh release, confirming other studies (Gundersen & Jenden, 1980). At concentrations that did not exert a post-junctional blockade, oxotremorine was unable to antagonize the presynaptic effects of Phen. In addition, previous incubation with atropine did not facilitate or prevent the increase of m.e.p.p. frequency induced by Phen, indicating that anti-muscarinic activity does not account for the enhanced spontaneous ACh release.

Alternatively, if the antinicotinic activity of Phen is responsible for the increase of m.e.p.p. frequency, this effect might be antagonized by increasing the concentration of ACh in the synaptic cleft with anticholinesterase agents. However, paradoxical results were obtained with neostigmine which potentiated the presynaptic effect of a low (20 μM), but not of a high (50 μM) concentration of Phen. One possibility is that at the higher concentration, the antagonist may have reached its maximal enhancing capability on the spontaneous ACh release, the reason why neostigmine did not potentiate the effect. On the other hand, an eventual potentiation of the pre-

junctional effect may have been missed with intracellular recording techniques, because of the intense depression of m.e.p.p. amplitudes induced by high concentrations of Phen.

The increase in m.e.p.p. frequency induced by Phen was not altered in the presence of tubocurarine, whereas the post-junctional blockade was exacerbated. Due to the latter effect, higher concentrations of tubocurarine could not be tested and therefore, the involvement of presynaptic nicotinic receptors cannot be ruled out. Studies using other techniques such as chemical determinations of the ACh release may provide better evaluation of the actions of both antagonists (Wessler *et al.*, 1986).

At concentrations that increased m.e.p.p. frequency, Phen did not influence the evoked transmitter release as shown by determinations of the quantal content of e.p.s. Equal concentrations of Phen did not alter the rundown of successively evoked e.p.s. as did tubocurarine (Figure 6). The faster rundown of the endplate current amplitudes recorded in the presence of tubocurarine have been attributed to its presynaptic actions (Glavinovic, 1979b; Magleby *et al.*, 1981; Gibb & Marshall, 1984). A faster decay of the e.p.p. amplitude was also induced by a high concentration of Phen (50 μM). Considering the intense postjunctional blockade obtained in this case, those results may not be explained solely by a presynaptic action.

In conclusion, the results showed that in the rat phrenic nerve diaphragm, Phen facilitated the spontaneous quantal release of ACh without altering the evoked transmitter release. The effect was not related to either the temperature or membrane depolarization nor did it depend on the $[\text{Ca}^{2+}]_o$. The presynaptic effect may be explained by an interaction of Phen with membrane-bound receptors since the agent does not cross the nerve terminal membrane. Our findings did not support an interaction with putative presynaptic muscarinic receptors; a possible interaction with presynaptic nicotinic cholinergic receptors could not be excluded due to the technical limitations of intracellular recordings.

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