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A comparison between mechanisms of action of different nicotinic blocking agents on rat submandibular ganglia

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1 The blocking properties of tubocurarine, decamethonium, hexamethonium and trimetaphan on nicotinic agonists applied by repetitive ionophoretic pulses were examined in rat submandibular ganglion cells using a two-microelectrode voltage-clamp technique at 30°C.

2 Hexamethonium, a proposed slowly dissociating, open-channel blocker at concentrations of 2–20 μM did not produce a 'use-dependent' run-down of responses, but its antagonism was clearly dependent on membrane potential.

3 The voltage-dependent reduction of agonist response by hexamethonium was not dependent on the nature of agonist used. Similar results were obtained with acetylcholine (ACh) and carbamylcholine (CCh) ionophoresis.

4 (+)-Tubocurarine (5 μM) and decamethonium (10 μM) produced 'use-dependent' run-down of agonist responses which became more pronounced at higher frequency and as the cell was hyperpolarized, consistent with open-channel blockade. In contrast, trimetaphan (2.5 μM), a receptor antagonist did not cause 'use-dependent' run-down of responses.

5 Hence, the antagonism produced by hexamethonium, unlike tubocurarine and decamethonium, could not be accounted for in terms of open-channel blockade but requires an alternative mechanism, the nature of which is discussed.

Introduction

Non-depolarizing ganglion-blocking agents (e.g. hexamethonium) have long been thought to produce their effect by a competitive action (Van Rossum, 1962). However, numerous studies (Blackman, 1970; Ascher *et al.*, 1979; Gurney & Rang, 1981; Selyanko *et al.*, 1981; 1982; Rang, 1982) suggest that hexamethonium might act by blocking the ionic channels opened as a consequence of agonist-receptor interactions.

In the kinetic studies on rat submandibular ganglion cells of Ascher *et al.* (1979), hexamethonium and tubocurarine appeared to behave as slowly dissociating open-channel blockers and decamethonium as a fast dissociating blocker. A consequence of drugs which dissociate slowly from ion-channels is the phenomenon of 'use-dependence' (Courtney, 1975); the level of blockade is enhanced as the frequency at which channels are opened increases. This phenomenon is of particular interest as it could account for the pronounced frequency-dependence shown by many ganglion-blocking agents (Paton & Zaimis, 1949; 1951; Kharkevich, 1967). However, unlike tubocurarine, hexamethonium produced a voltage-dependent reduction of the 'instantaneous' current following a hyperpolarizing jump (Ascher *et al.*, 1979) or of synaptic currents (Rang, 1982) in rat ganglion cells which would not be predicted for open-channel blockade.

The aim of the present study was to investigate the action of hexamethonium in terms of 'use-dependence' and voltage-dependence and compare it with those of other ganglion-blocking drugs. The main finding suggests that the antagonism produced by hexamethonium and trimetaphan is different from that of tubocurarine and decamethonium. The latter

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two appeared to behave as, respectively, slow and fast dissociating open-channel blockers. A preliminary account of some of these results has been published (Large & Sim, 1984).

Methods

The methods used were similar to those described by Ascher *et al.* (1979). Rats (150–200 g, Wistar strain of either sex) were killed with excess chloroform and bled out by injection of Krebs solution into the left ventricle. The submandibular ganglia, with adjacent stretches of the lingual nerves and salivary ducts, were excised and placed in Krebs solution (mM: NaCl 119.0, KCl 4.7, KH_2PO_4 1.2, $MgSO_4$ $7H_2O$ 1.2, glucose 11.0, $NaHCO_3$ 25.0, $CaCl_2$ 2.5) equilibrated with 95% O_2 and 5% CO_2, pH 7.4. The ganglion was pinned over a small pad of Sylgard resin in the recording chamber (volume about 1 ml) and as much connective tissue as possible was removed. The recording chamber was then mounted on the stage of a Nomarski microscope (fitted with a water immersion ×40 objective) and was superfused continuously with oxygenated Krebs solution at 3 ml min^{-1}; the temperature was maintained at 30°C.

The voltage clamp circuit was similar to that described by Dionne & Stevens (1975). Micro-electrodes were pulled from 1.0 mm glass containing an internal filament (Clark Electro-medical) on a Narishige horizontal puller. Recording electrodes were filled with 4 M potassium acetate and had resistance of 100–150 MΩ. Current-passing electrodes were filled with 0.5 M potassium sulphate and had resistances of 80–150 MΩ. The current signals were passed through a low pass filter (Barr and Stroud, type EF-3) with a cut-off set to 200–400 Hz. During drug application and washout, the cell was held at −50 mV, close to the resting membrane potential of cells impaled with two microelectrodes.

Nicotinic agonists were applied onto the ganglion cells by ionophoresis. Ejecting and retaining currents were generated by a current pump similar to that described by Dreyer & Peper (1974). Ionophoretic pipettes were filled with either 1 M carbamylcholine chloride (pH 5.0) or 2 M acetylcholine bromide (pH 4.9) and had resistances of 80–130 MΩ. A retaining current of about 1 nA and ejecting current of 2–4 nA were normally used. Care was given to the positioning of ionophoretic pipettes in order to elicit sharp responses.

Antagonists were bath-applied and at least 10 min were allowed for equilibration.

All signals were recorded directly by means of a potentiometric recorder and were simultaneously stored on magnetic tape (Racal Store 4 FM) for later analysis. A transient recorder (Datalab DL901) and an X/Y plotter (Bryans) were used to produce permanent records.

Drugs

The following pharmacological agents were used: acetylcholine bromide (Koch-Light Lab), carbamylcholine chloride (carbachol, Sigma), decamethonium iodide (Koch-Light Lab), hexamethonium chloride (Sigma), trimetaphan camsylate (Roche), (+)-tubocurarine chloride (Sigma).

Results

Agonist ionophoresis

In normal Krebs solution, nicotinic agonists were applied by brief (2–10 ms) ionophoretic pulses at various frequencies: 1 Hz for 10 s, 2 Hz for 5 s, 5 Hz for 2 s and 10 Hz for 1 s, at holding potentials of −50 mV and −80 mV. In most cases, 9 responses were evoked, the interpulse duration varying from 1 s at the lowest frequency to 100 ms at the highest. As shown in Figures 1 and 3, the amplitude of the control responses remained relatively constant at 1 Hz and 10 Hz at −50 mV and −80 mV in normal Krebs solution. The intensity of the ionophoretic pulse was adjusted to produce responses of relatively small amplitudes (0.2–1.5 nA at −50 mV), so as to minimize the effects of desensitization. Assuming that each channel has a linear voltage-current relationship (Anderson & Stevens, 1973; Dionne & Stevens, 1975) and that the reversal potential for a cholinoceptor agonist is −10 mV (Rang, 1981), the ratio of I (−80 mV)/I (−50 mV) (I = agonist-induced current) should equal 1.75. In the present experiments, the ratio was observed to be 1.76 ± 0.09 (mean ± s.e.mean) ($n = 24$) for carbachol (CCh) and 1.77 ± 0.09 ($n = 19$) for acetylcholine (ACh).

Effect of hexamethonium

Figure 1 shows the effect of hexamethonium (10 μM) on the amplitude of the currents induced by ionophoresis of CCh. There are two important features of the antagonism produced by hexamethonium. Firstly, the inhibition produced by hexamethonium was clearly voltage-dependent as responses were reduced ~58% at −50 mV compared to control and by ~82% at −80 mV (Figure 1). Secondly, the inhibition of hexamethonium was independent of the rate at which agonist was applied where the degree of inhibition at all frequencies was $58 \pm 6.0\%$ ($n = 6$) at −50 mV and $82 \pm 8.3\%$ at −80 mV (Figure 2). Similar results were obtained with ionophoresis of ACh, where the responses were reduced by $60 \pm 3.2\%$

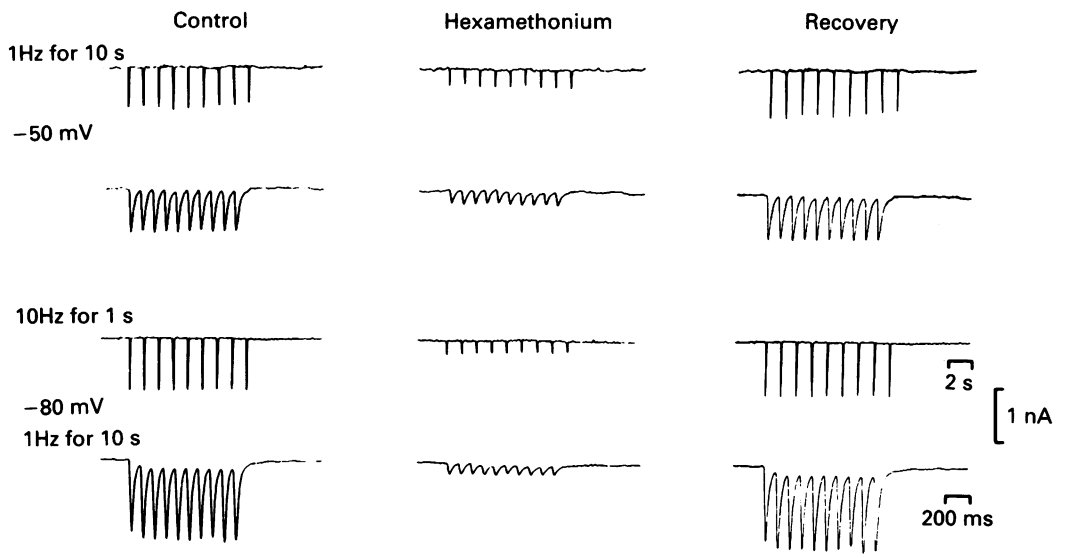


Figure 1 Effects of hexamethonium on the amplitudes of currents evoked by carbamylcholine (CCh) ionophoresis applied at 1 Hz and 10 Hz in a single cell. Currents were recorded in normal Krebs at −50 mV and −80 mV, and then after 10 min in the presence of hexamethonium (10 μM). The currents evoked were reduced by about 58% at −50 mV and 82% at −80 mV at both frequencies. The effect of hexamethonium was readily reversible after 15 min washout.

at all frequencies at −50 mV and by 82 ± 4.5% ($n = 4$) at −80 mV, illustrating that the antagonism by hexamethonium was not dependent on the nature of agonist used.

Effect of other ganglion-blocking agents

Trimetaphan (2.5 μM), a receptor antagonist, reduced the amplitude of agonist-induced currents equally, about 42 ± 5.6% ($n = 6$), within a train of stimuli and at all frequencies (Figure 3). Furthermore, the action of trimetaphan was not dependent on the holding potential, as an inhibition of 43 ± 6.2% ($n = 6$) was also observed at −80 mV at all frequencies studied (Figure 3). Similar results were obtained using ACh as the agonist.

Figure 4 illustrates the effect of tubocurarine (5 μM) on the amplitude of the currents evoked by CCh ionophoresis at 1 Hz and 10 Hz. Tubocurarine usually had a negligible effect on the first pulse occurring within a train (Figure 4) at either 1 Hz or 10 Hz frequency, but the degree of inhibition became gradually more pronounced with the consecutive responses within the train as shown with 10 Hz (Figure 4).

This 'use-dependent' run-down was dependent on the rate of agonist application, being scarcely apparent at 1 Hz but becoming more marked at 10 Hz (Figure 4). The antagonism produced by tubocurarine was dependent on the holding potential, being more pronounced as the cell was hyperpolarized to −80 mV. The last response at 10 Hz was reduced by 65 ± 2.8% at −80 mV compared to 35 ± 2.8% at −50 mV ($n = 6$).

Decamethonium (10 μM) caused a voltage-dependent reduction of the first pulse within the train, being 13 ± 1.6% at −50 mV and 26 ± 0.8% at −80 mV ($n = 6$), at all frequencies. In addition, decamethonium also displayed a degree of 'use-dependent' reduction of the subsequent pulses within a train. Similar to tubocurarine, the 'use-dependent' effect of decamethonium was dependent on the rate of agonist ionophoresis as well as the holding potential (Figure 5).

The 'use-dependent' action of both tubocurarine and decamethonium showed a dependence on the nature of agonist used, being more marked with ACh than with CCh: e.g. the last pulse of a 10 Hz ACh train at −80 mV was reduced by 75 ± 2.0% ($n = 6$) which was significantly greater ($P < 0.025$) than reduction in a CCh train by 65 ± 2.8% with tubocurarine; and by 75 ± 3.1% ($n = 6$) with ACh compared to 60 ± 4.3% with CCh ($P < 0.02$) in the presence of decamethonium.

Does hexamethonium produce a 'closed-blocked' channel?

Figure 6 shows the results of an experiment designed to test whether the lack of 'use-dependence' displayed by hexamethonium could be accounted for in terms of

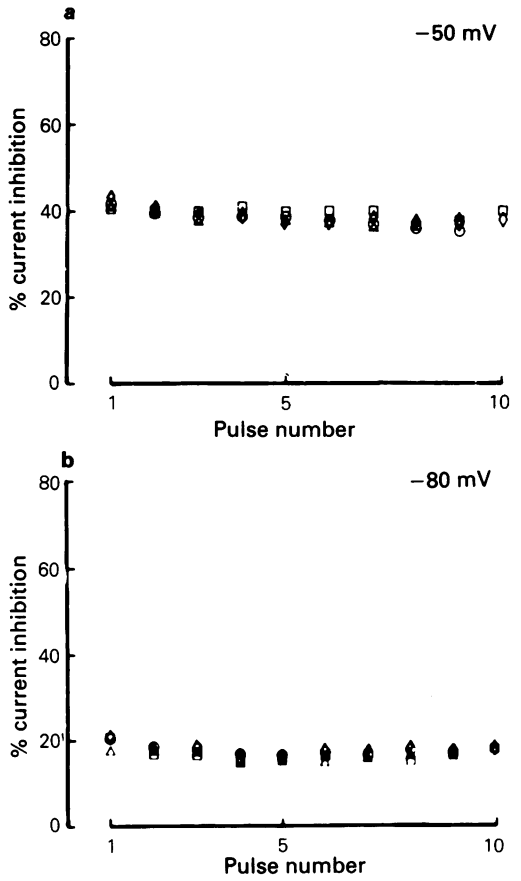


Figure 2 The effect of hexamethonium (10 μM) on trains of carbachol (CCh)-induced currents. CCh was applied at 1 Hz for 10 s (○), 2 Hz for 5 s (□), 5 Hz for 2 s (△) and 10 Hz for 1 s (◇) at holding potentials of (a) − 50 mV and (b) − 80 mV. The responses are expressed as percentage of control and plotted against pulse number. Each point represents the mean of 6 experiments and the s.e.mean was always less than 10%. Note the voltage-dependent effect of hexamethonium.

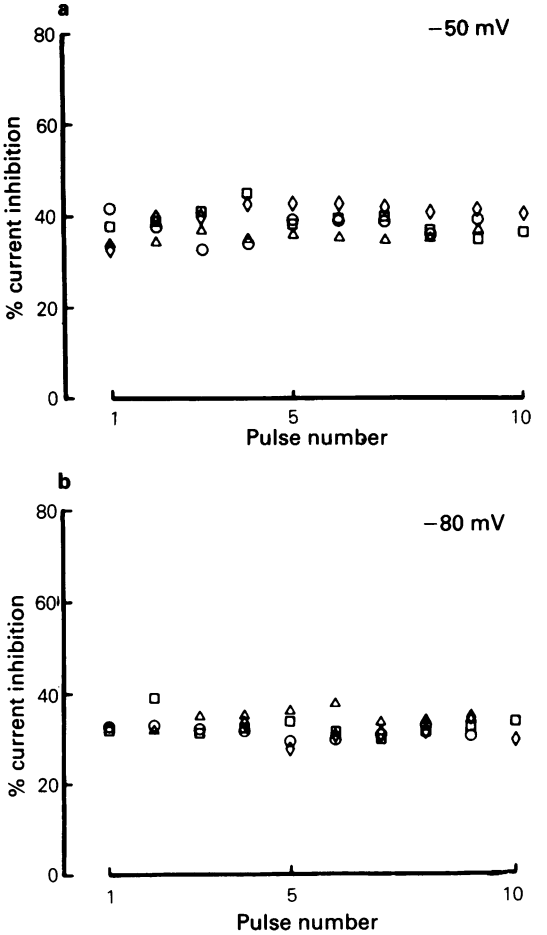


Figure 3 Effect of trimetaphan (2.5 μM) on trains of carbachol (CCh)-induced currents. CCh was applied at 1 Hz (○), 2 Hz (□), 5 Hz (△) and 10 Hz (◇) at (a) − 50 mV and (b) − 80 mV. The responses are expressed as percentage of control and plotted against pulse number. Each point depicts the mean of 4 experiments and the s.e.mean was always less than 10%. Note, the voltage insensitivity of trimetaphan and the lack of run-down.

hexamethonium molecules becoming trapped within the channels as they close. With this type of antagonism by positively charged blockers, unblocking of the channels can be achieved by re-exposure to the agonist at more depolarized potentials. The first pulse, on return to more negative potential, will be expected to show recovery from the 'closed-blocked' state. In addition, after washout of the antagonist, channels which are still in the 'closed-blocked' state would only show recovery on re-exposure to agonist. However, no recovery was observed with re-exposure to agonist at − 30 mV, even at high frequency ionophoresis, in the continuing presence of hexamethonium (2 μM) (Figure 6). In addition, the action of hexamethonium, as with the other ganglion-blocking agents studied, was readily reversed after 10 min washout without further exposure to agonist.

Dose-response experiments on voltage-clamped ganglion cells

A further comparison of the actions of hexamethonium and decamethonium was made using dose-response curves. Curves were constructed using brief (2–20 ms) pulses of increasing amplitudes (2–50 nA) on voltage-clamped ganglion cells. Figure 7 shows the

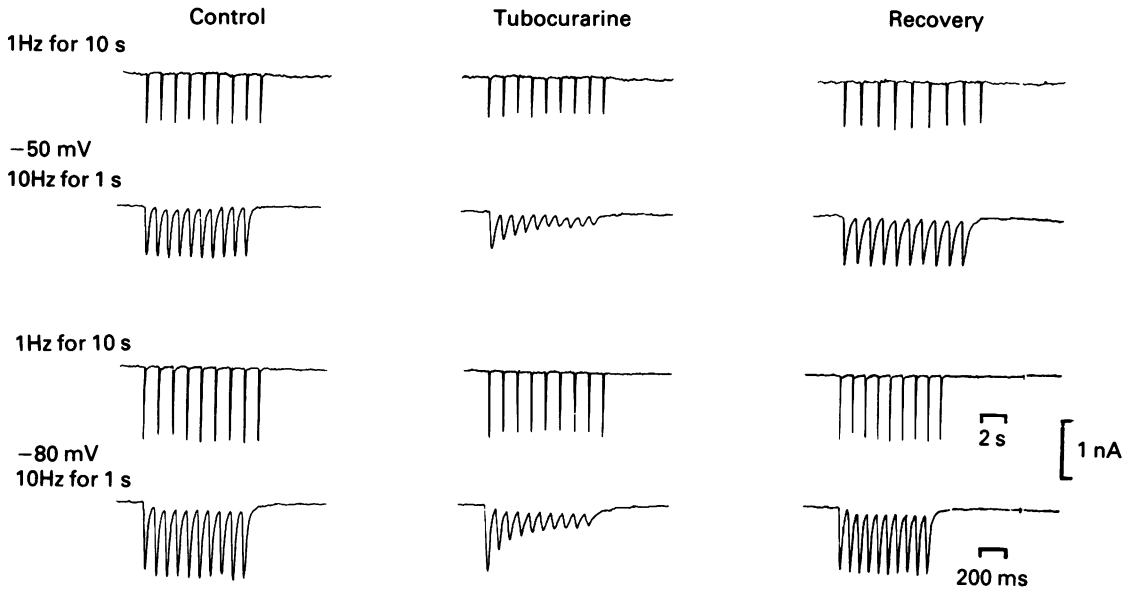


Figure 4 The 'use-dependent' effect of tubocurarine on the amplitudes of currents evoked by carbachol (CCh) ionophoresis at 1 Hz and 10 Hz in a single cell. Currents were recorded in normal Krebs at −50 mV and −80 mV, and then after 10 min in 5 μM tubocurarine. Tubocurarine had a negligible effect at 1 Hz at both holding potentials. At 10 Hz, the first pulse within the train was little affected by tubocurarine and the subsequent responses were reduced in a 'use-dependent' manner. The action of tubocurarine was completely reversible after 15 min washout.

dose-response curves in the absence and presence of decamethonium or hexamethonium in a single cell. Once the control dose-response curves had been established, bath-applied antagonists were allowed to equilibrate for at least 10 min at −50 mV, before returning the potential to −80 mV. In the presence of decamethonium (50 μM) the dose-response curve was flat (Figure 7a) as would be expected for an open-channel blocker. The action of decamethonium was reversed on washout and control dose-response curves were re-constructed at −80 mV. On addition of hexamethonium (20 μM), the dose-response curve was shifted to the right in an unparallel fashion (Figure 7b) and no flattening of the dose-response curves was observed with concentrations up to 40 μM. Although it can be argued that this mode of analysis has the weakness that the relationship of agonist concentration to ionophoretic charge is unknown and that the area of receptive surface activated may change, such studies serve to illustrate that the antagonism of hexamethonium is clearly different from that shown by a supposed open-channel blocker.

Discussion

The results with hexamethonium in respect of 'use-dependence' did not conform to the predictions for a slowly dissociating open-channel blocker. On the other hand, using similar protocols, the open-channel blocking properties of tubocurarine and decamethonium were clearly demonstrated, along with the receptor antagonist action of trimetaphan in rat submandibular ganglia (Table 1). Thus, tubocurarine produced a frequency- and voltage-dependent blockade consistent with an action on open-channels, as proposed by Ascher *et al.* (1979) and Rang (1982). The effects of trimetaphan, which showed a voltage insensitivity and a lack of 'use-dependence' are consistent with an interaction with the receptors (Ascher *et al.*, 1979; Rang, 1982).

Decamethonium reduced the peak amplitude of agonist-induced responses in a voltage-dependent manner as would be expected for a fast dissociating channel blocker (Ascher *et al.*, 1979), though its 'use-dependence' could not be predicted. However, the complex action displayed by decamethonium may have resulted from fast dissociating channel block (Ascher *et al.*, 1979) on the mixture of channel types in this preparation (Rang, 1981; 1982).

The unexpected lack of 'use-dependence' displayed by hexamethonium was not consistent with its proposed open-channel blocking property (Ascher *et al.*, 1979; Gurney & Rang, 1981; Rang, 1982). In all 42

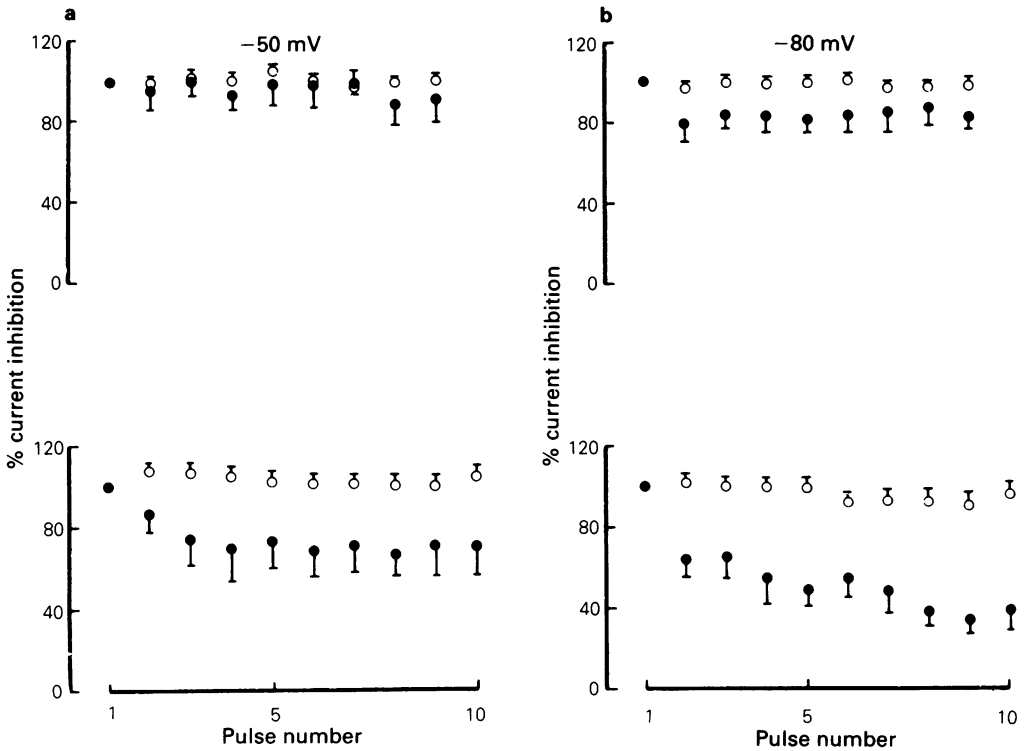


Figure 5 'Use-dependent' effect of decamethonium (10 μM) on trains of carbachol (CCh)-induced currents. CCh was applied ionophoretically at 1 Hz (top traces) and 10 Hz (lower traces) at holding potentials of −50 mV (a) and −80 mV (b). To express the 'run-down' effect of decamethonium, without the added voltage-dependent reduction, the amplitudes of the responses are expressed as percentage of the first pulse and plotted as a function of the pulse number, in normal Krebs solution (●) and in the presence of 10 μM decamethonium (○). Each point denotes the mean of 6 experiments with vertical lines showing s.e.mean.

cells studied in the presence of hexamethonium, no evidence of 'use-dependence' was ever recorded with agonist applied at a frequency of 10 Hz.

Gurney & Rang (1984) described a 'use-dependent' run-down with hexamethonium, but a 'run-down' of the peak amplitudes was also observed in the absence of the antagonist. It is plausible that such a discrepancy could arise from the size of agonist-induced responses used in the present study, although the use of 'relatively' larger CCh responses (Figure 8) did not display any phenomenon of 'use-dependence'; it was not possible to increase the ionophoretic pulses further as marked run-down of peak amplitude was observed in normal Krebs (cf. Sim, 1984). Therefore, it is important to use relatively small ionophoretic responses of equal amplitudes in the characterization of drugs in terms of 'use-dependence'.

The action of hexamethonium appeared to be independent of the nature of agonist used, as similar results were obtained with CCh and with ACh, the latter having a longer channel open-time than CCh (Gray & Rang, 1983). In contrast, tubocurarine and decamethonium displayed greater antagonism against ACh than against CCh, as would be predicted from the longer channel open-time evoked by ACh.

Ascher *et al.* (1979) observed slow 'outward' relaxation currents following hyperpolarizing jumps in the presence of tubocurarine and hexamethonium, indicative of slowly dissociating channel blockers (cf. Ascher *et al.*, 1978), though the 'outward' relaxation current recorded with hexamethonium is usually small (Large, unpublished observation). In contrast, 'inward' relaxation current recorded in the presence of decamethonium (Ascher *et al.*, 1979) was consistent

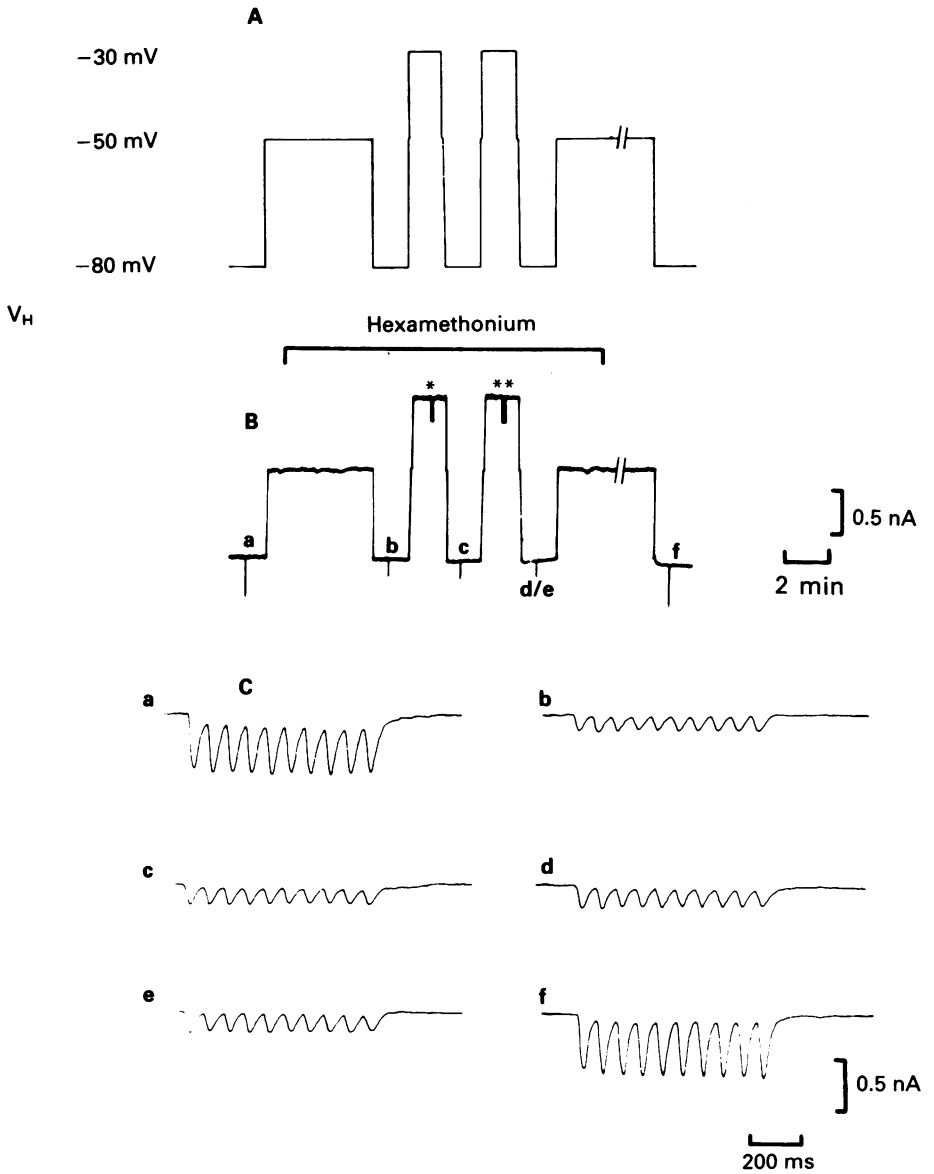


Figure 6 The effect of frequency of agonist ionophoresis and membrane potential on the antagonism of hexamethonium. (A and B) Illustrate the sequence of an experiment where carbachol (CCh) was tested at 10 Hz for 1 s and at −80 mV holding potential. The lower traces denote the recorded responses at 10 Hz for 1 s at the corresponding positions of the upper traces. At −80 mV, a control test train of 10 Hz was elicited (a), and the potential was returned to −50 mV. The addition of hexamethonium (2 μM) for 5 min, preceded returning the potential to −80 mV and a further test train (b). *The potential was changed to −50 mV then −30 mV* where CCh was applied at 10 Hz for 5 s. The potential was returned to −80 mV, then a test train elicited (c). No recovery in amplitude was observed. The procedure *−* was repeated and CCh was now applied at 10 Hz for 10 s. The potential was returned to −80 mV, then a test train elicited (d and e). The potential was returned to −50 mV where hexamethonium was washed out for 15 min before a test train at −80 mV was elicited (f). The effect of hexamethonium was completely reversed on washout without further exposure to CCh ionophoresis.

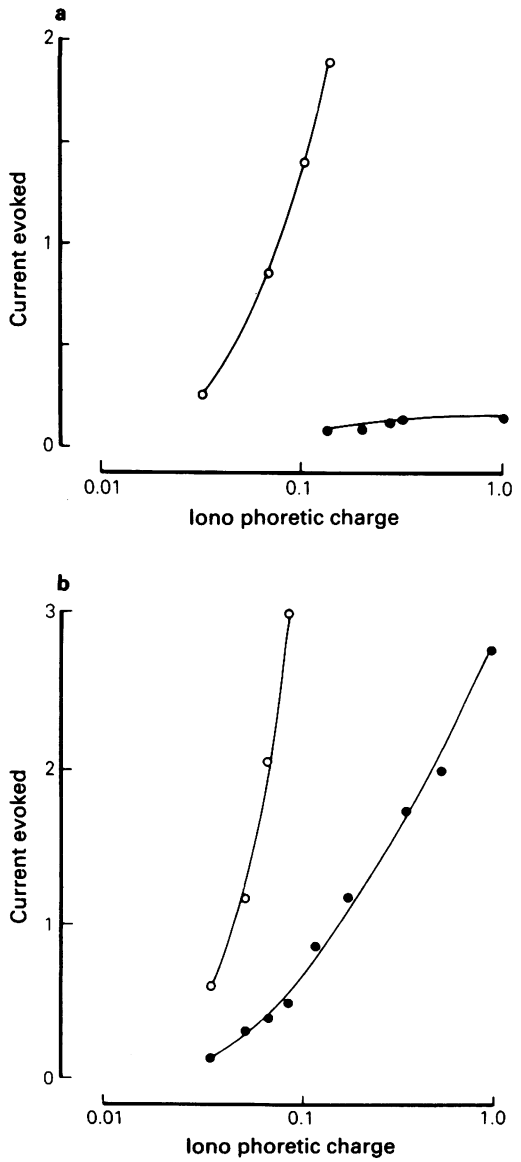
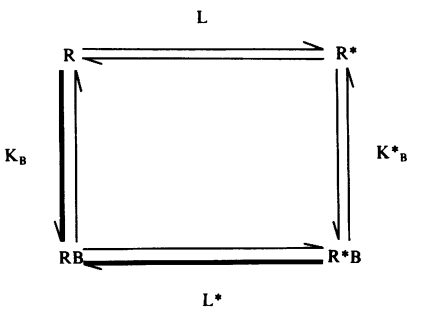


Figure 7 Dose-response curves to carbachol (CCh) applied ionophoretically were obtained in Krebs solution (○) and in the presence of antagonists (●) in a single cell held at −80 mV. (a) In the presence of decamethonium (50 μM) which induced a flattening of the curve. (b) In the presence of hexamethonium (20 μM). Increasing the ionophoretic pulse current overcame the antagonism.

with a fast dissociating channel blocking action (Marty, 1978). Thus, hexamethonium does not behave as a fast dissociating channel blocker, and our own results also indicated that its action is not comparable to that of decamethonium.

Voltage-dependent inhibition of the peak amplitude of ACh responses, evoked synaptically or ionophoretically, by hexamethonium has previously been described in autonomic ganglia (Ascher *et al.*, 1979; Selyanko *et al.*, 1981; 1982; Rang, 1982; Gurney & Rang, 1984). Such an action could not be accounted for in terms of the sequential model of open-channel blockade (cf. Rang, 1982) and a secondary action of hexamethonium was postulated. A plausible hypothesis is that hexamethonium binds to a site close to the mouth of the channel and, once it is opened, the antagonist molecule moves to a site deep within it (Rang, 1982). An extension of this is for the channel then to close, trapping the hexamethonium molecule within (Gurney & Rang, 1984), as described for the action of chlorisondamine at the gastric mill muscle of the lobster (Lingle, 1983a). 'Trapping' can be overcome by further exposure to agonist at more depolarized potentials or during washout of antagonist (Lingle, 1983a,b). However, in the present study, no evidence of 'trapping' of hexamethonium could be demonstrated (Figure 6) and the action of hexamethonium could be reversed without further exposure to agonist.

Therefore, from the present results, we propose an action of hexamethonium on channels in the closed state. Such an action can be represented in terms of a cyclic model analogous to that proposed by Adams (1976) for the action of barbiturates as follows:



where R and R* depict the closed and open states of the channels, respectively. RB is the closed but blocked state and R*B the open but blocked state, B is the antagonist, K_B, K^*_B and L,L* represent dissociating and conformation equilibrium constants respectively.

An action on channels in the closed state predicts that $L < L^*$ and consequently $K_B < K_B^*$ (i.e. the speed

Table 1 Summary of the antagonist properties of the various ganglion-blocking agents on trains of carbachol applied ionophoretically

Antagonists	*Effect on first pulse*	*Voltage-dependence*	*Frequency-dependence*	*Agonist-dependence*
(+)-Tubocurarine	−	+*	+	+
Decamethonium	+	+*†	+	+
Hexamethonium	+	+†	−	−
Trimetaphan	+	−	−	−

*Refers to the voltage-dependence of the run-down effect.
†Refers to the voltage-dependence on peak amplitude of responses.

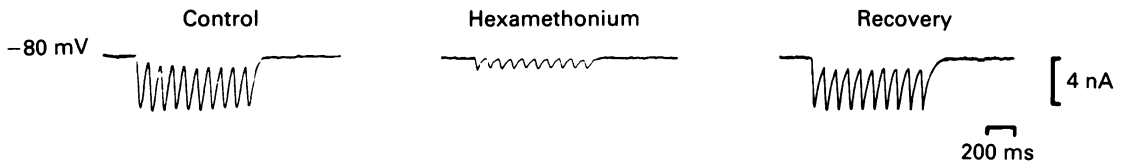


Figure 8 The effect of hexamethonium on a train of currents elicited with larger ionophoretic current pulses. Carbachol (CCh) was applied ionophoretically at 10 Hz to a single cell. The train of currents was recorded in normal Krebs, then after 10 min in 10 μM hexamethonium. Note, no run-down was observed and there was complete recovery.

with which closed channel block adjusts to changes in membrane potential requires the rate of association and dissociation of the blocker to be faster than seen with open-channel block). In addition, the model also predicts that the time course of agonist-induced responses would be shortened in the presence of antagonist ($L^* > L$). However, it is not possible to carry out a detailed kinetic study to establish the proposed action of hexamethonium on closed state channels using ionophoresis.

It would be feasible to investigate such a mode of antagonism using single channel recording techniques where fast and slow types of channels (Rang, 1981) can be distinguished. Until such studies are made, the proposed action of hexamethonium on closed state channels remains speculative.

In conclusion, the phenomenon of 'use-dependence' serves as a useful pharmacological tool, not only in distinguishing drugs which act at the level of ion-channels but also at the receptor level. Moreover, it reveals an additional property of hexamethonium which was clearly not consistent with the proposed open-channel blockade at the rat submandibular ganglia.

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