Modes of hexamethonium action on acetylcholine receptor channels in frog skeletal muscle

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- 1 The antagonism between hexamethonium and cholinergic agonists was investigated in frog skeletal muscle fibres with voltage-clamp techniques. Hexamethonium caused a voltage-dependent reduction in the amplitude of endplate currents. For neurally evoked endplate currents, the reduction increased e-fold with a 38 mV membrane hyperpolarization.
- 2 The effect of hexamethonium on the time course of endplate currents was small, and was most apparent as a slight prolongation of the decay phase at hyperpolarized potentials (more negative than –100 mV). A similar small prolongation of single channel lifetime was detected with fluctuation analysis techniques. Hexamethonium produced a voltage-dependent reduction in apparent single channel conductance as the membrane was hyperpolarized.
- 3 Log (concentration-response) curves for acetylcholine (ACh)-induced currents, determined either from currents accompanying ramp changes in membrane potential or from steady state currents in voltage-jump experiments, were less steep for responses in the presence of hexamethonium. This reduction in slope became more pronounced at more negative membrane potentials. Observations at +50 mV suggested that the equilibrium constant for competitive antagonism was approximately 200 μ M.
- 4 In voltage-jump experiments with a two-microelectrode voltage clamp, the current evoked by ACh in the presence of hexamethonium differed from that recorded with ACh alone. In the presence of hexamethonium, the expected 'instantaneous' ohmic increase in membrane current in response to a hyperpolarizing step was not detected; instead a decrease in current was observed. This problem was further investigated with a vaseline-gap voltage-clamp technique which provides improved temporal resolution. With this method a rapid decrease in the ACh-induced inward current was observed with step hyperpolarizations in the presence of hexamethonium.
- 5 When the membrane potential was stepped back to its resting level from a more hyperpolarized potential in the presence of hexamethonium, there was a surge of ACh-induced inward current that decayed with a time constant of less than 100 μ s.
- 6 The slow relaxation in the ACh-induced current that followed a voltage step recorded in the presence of hexamethonium was slower than that recorded with ACh alone. In the presence of hexamethonium the time constant of this relaxation increased e-fold for a 67 mV hyperpolarization.
- 7 The results are consistent with a rapid voltage-dependent block of ACh-activated channels by hexamethonium with hyperpolarization, and voltage-dependent unblock with depolarization. The voltage-dependent block is combined with competitive antagonism at the ACh receptors. However, not all observations appear to be compatible with a simple sequential block of open ion channels, but rather suggest that occupation of the channel by hexamethonium may not prevent channel closure.

Introduction

Hexamethonium has been known since the experiments of Paton & Zaimis (1949) to block transmission both at autonomic ganglia and at the neuromuscular junction. The principle factor in the blocking action at ganglia has been shown to be a block of ion channels associated with acetylcholine (ACh) receptors rather than a competitive antagonism at the receptors (Blackman, 1959; Ascher *et al.*, 1979; Rang, 1982; Skok *et al.*, 1983; Gurney & Rang, 1984). At the neuromuscular junction, where hexamethonium is less potent, the action of the drug is less well understood. From investigations of the interactions between tubocurarine and hexamethonium at the rat neuromuscular junction Ferry & Marshall (1973), Blackman *et al.* (1975) and Rang & Rylett (1984) concluded that the two drugs interacted at the same sites and that at least a part of the action of hexamethonium was at ACh receptors. Milne & Byrne (1981) investigated the effects of

hexamethonium on endplate currents in frog muscle in which neuromuscular transmission had been blocked with high concentrations of magnesium and concluded that hexamethonium blocked open ion channels. More recently, Brenner & Micheroli (1985) concluded from a study on normal and vagus-reinnervated frog endplates that hexamethonium acted both as a competitive antagonist and as a channel blocking agent, although direct evidence for channel block could not be demonstrated.

We have investigated the effects of hexamethonium on neurally evoked endplate currents and have examined the interaction between bath applied agonists and hexamethonium. The major effect of hexamethonium was to reduce the amplitude of endplate currents in a voltage-dependent manner. From observations on the relaxations of ACh-induced currents evoked by voltage steps, we conclude that hexamethonium blocks ion channels associated with ACh receptors. The observations cannot, however, be accounted for by a simple sequential block of open ion channels of the type previously advanced for local anaesthetic block of ACh-operated channels (Ruff, 1977; Neher & Steinbach, 1978). One possibility consistent with the results is that hexamethonium can remain in the channel without substantial inhibition of the normal process of channel closure, in the way suggested for hexamethonium at ganglion cells (Gurney & Rang, 1984).

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A preliminary account of some of these results has been presented to the British Pharmacological Society (Adams *et al.*, 1983).

Methods

Intracellular microelectrode experiments

The initial series of experiments was made on endplates of the cutaneous pectoris muscle of *Rana temporaria*. Endplate regions were located using Nomarski optics; the location was confirmed by focal recording of miniature endplate potentials (m.e.p.ps) with an intracellular electrode filled with 3M KCl. The endplate membrane was voltage clamped with a second (current passing) microelectrode filled with 2M K acetate inserted adjacent to the voltage recording electrode. Current was measured either as the voltage drop across a 1 M Ω resistor in series with the current-passing electrode or with a virtual earth current to voltage converter.

Muscles were bathed in a Ringer solution of the following composition (mM): NaCl 115, KCl 2, CaCl₂ 1.8, buffered to pH 7.2 with either Na-HEPES 5 or Na₂HPO₄ 1.92 plus NaH₂PO₄ 0.48. To minimize electrode displacement upon muscle contraction, muscles were stretched to 1.5 times their resting length (Terrar, 1978). Tetrodotoxin (100 nM) was added to the external solution except when neurally evoked e.p.ps were under investigation. In some experiments a high magnesium Ringer solution was used; this contained no added calcium and 10 mM MgCl₂. For dose-response experiments, muscles were superfused with Ringer solution containing either carbamylcholine chloride (CCh, Sigma) or acetylcholine chloride (ACh, Sigma). In these experiments acetylcholinesterase was inhibited by the addition of neostigmine methylsulphate 3 μ M to the external solutions. Hexamethonium bromide (Sigma) was added to the Ringer solution, at the concentration stated. Experiments were usually carried out at room temperature (20–23°C), although in some experiments the temperature was lowered to 13°C.

Endplate currents were recorded on FM tape (Racal Store 4) at 7½ ips (bandwidth d.c. to 2.5 kHz) and later photographed. In some experiments agonist induced-current during changes of membrane potential was determined by subtracting current in the absence of agonist from that in its presence. This was done both for experiments where the membrane potential was changed gradually by applying 'ramp' commands to the voltage-clamp, and in other experiments where the membrane potential was stepped from one potential to another ('voltage-jumps'). In some voltage-jump experiments a Neurolog NL750 averager was used to give the average current during a number of similar changes in membrane potential, and thus to increase the signal-to-noise ratio.

Vaseline-gap voltage clamp experiments

Acetylcholine-induced current fluctuation and current relaxation measurements were made on single muscle fibres dissected from innervated and denervated (14–21 days) semitendinosus muscles of *Rana temporaria*. Fibres were voltage-clamped by the vaseline-gap technique as previously described (Hille & Campbell, 1976; Adams *et al.*, 1981). The ends of the muscle fibres were cut in an unbuffered 'internal' solution of 80 mM K EGTA (pH 7.2) and the A-pool (volume 0.1 ml), which contained the endplate membrane or extra-junctional region of denervated fibres, was continuously perfused at >200 μ l s⁻¹ with control (normal Ringer) or agonist-containing external solutions. The external Ringer solution used for these experiments contained (mM) NaCl 114, KCl 2.4, CaCl₂ 1.5, Na-HEPES buffer 10 pH 7.2. The effective rate of exchange of the external solution composition at the membrane face was approximately 1.5 s⁻¹ (Adams & Colquhoun, unpublished). The cholinomimetic agonists, acetylcholine chloride (Sigma) and suberyldicholine diiodide, (kind

gift of Dr B. Sakmann) were bath-applied to active ACh receptor-channels.

Analysis of both current fluctuations and relaxations was done by the methods described by Colquhoun *et al.* (1979) and Colquhoun & Sheridan (1981). For acetylcholine-induced current fluctuations, high gain a.c. current records were filtered through an eight-pole Butterworth bandpass filter (Barr & Stroud, EF3-02) set at 0.05 Hz and 1 kHz. Net power spectra, obtained from the difference of spectra recorded in the presence and absence of agonist, were calculated at 0.5 Hz resolution and fitted by a least-squares fitting routine on LSI 11/03 computer. The routine calculated estimates of the parameters of the spectra and the standard deviations of these estimated parameters.

A PDP 11/40 computer with a laboratory interface (Cambridge Electronic Design 502) was used to supply command potentials for the vaseline-gap voltage-jump experiments, each voltage-jump being followed by on-line sampling of endplate currents, filtered at between 2 and 80 kHz (–3 dB point, four-pole Bessel filter). Details of the sampling and fitting of current relaxations are described by Colquhoun & Sheridan (1982). The reversal (zero-current) potential for open ACh receptor-channels was obtained by direct measurement and occasionally used to constrain the fit of the relaxation(s) to intercept the estimated instantaneous (zero-time) current. The membrane potential, clamp current and variance of the current fluctuations (rms noise) were monitored throughout experiments with a flat-bed chart recorder (Kipp & Zonen, BD 41).

All vaseline-gap experiments were carried out at a maintained temperature of 10–12°C, as measured by a thermistor probe located close to the muscle fibre.

Results

Effects of hexamethonium on the amplitude of endplate currents

Figure 1 shows endplate currents recorded before (a1 to a3) and after 3 min exposure to 1 mM hexamethonium (b1 to b3) at three membrane potentials, –60 mV, –90 mV and –120 mV in an external solution containing physiological concentrations of calcium and magnesium. It can be seen that hexamethonium reduced the amplitude of endplate currents at –60 mV (a1,b1) and that the extent of this reduction was greater when the fibre was hyperpolarized to –90 mV (a2,b2) or –120 mV (a3,b3). This voltage-dependent reduction in the amplitude of the endplate currents by hexamethonium is illustrated graphically in Figure 2 where endplate current amplitude is plotted against membrane potential (squares before, circles during hexamethonium). To assess the voltage-dependence of the reduction in peak amplitude of endplate currents, the value of $R-1$ for various membrane potentials was calculated (Acher *et al.*, 1979) where

$$R = \frac{\text{amplitude of control e.p.c.}}{\text{amplitude of e.p.c. in pres. of hexamethonium}}$$

Based on results like those shown in Figure 1, an e-fold change in $R-1$ was produced by a 38 mV change in membrane potential. This is similar to the reduction of synaptic current amplitude by hexamethonium in rat submandibular ganglia (Rang, 1982) and at the rat neuromuscular junction (Rang & Rylett, 1984). Voltage-dependent antagonism of this kind was consistently seen in all fibres studied with hexamethonium concentrations in the range of 100 μ M to 1 mM.

Hexamethonium also reduced the amplitude of the current evoked by bath-applied carbachol in a voltage-dependent manner. This is illustrated in Figure 3 which shows the results of an experiment in which the membrane potential at the endplate of a voltage-clamped muscle fibre was made to follow a slow ramp command which depolarized or hyperpolarized the

Figure 1 Effect of hexamethonium on the amplitude of endplate currents recorded from one muscle fibre before (a1, a2 and a3) and in the presence of hexamethonium, 1 mM, applied in the solution bathing the muscle for at least 3 min (b1, b2 and b3). Membrane potential: -60 mV (a1, b1); -90 mV (a2, b2); or -120 mV (a3, b3).

membrane to potentials between -60 mV and -120 mV. Current measurements were made in the absence and presence of carbachol and the difference between the currents was taken as the carbachol-induced current. At least two doses of carbachol were tested in this way; the complete procedure was then repeated for several carbachol concentrations in the presence of hexamethonium ($100\text{ }\mu\text{M}$ to 1 mM). For the example in Figure 3, while the carbachol-induced current increased with hyperpolarization in the absence of hexamethonium, the agonist-evoked current decreased with hyperpolarization when hexamethonium was present.

Log(concentration)-response curves to carbachol in the absence and presence of hexamethonium were determined from currents at three levels of membrane potential, -60 mV, -90 mV and -120 mV, and examples are shown in Figure 4. It can be seen that the log(concentration)-response curve for carbachol at -120 mV was much less steep in the presence

than in the absence of hexamethonium, and that the flattening effect of hexamethonium on the carbachol log(concentration)-response curve was reduced at less negative membrane potentials.

To avoid diffusion problems associated with the presence of the nerve terminal and the narrow synaptic cleft, equilibrium responses were also studied at extrajunctional regions of single denervated muscle fibres. Responses to bath applied $10\text{ }\mu\text{M}$ ACh were recorded in the presence and absence of $200\text{ }\mu\text{M}$ hexamethonium at membrane potentials of $+50$ mV and -50 mV (Figure 5). In this experiment, the peak amplitude of the agonist-induced response (145 nA) at $+50$ mV was reduced to 45% of control by $200\text{ }\mu\text{M}$ hexamethonium, while at -50 mV, the response was reduced to 15% of the control value (-172 nA). At a membrane potential of $+50$ mV, the influence of the electrochemical gradient to drive hexamethonium into the open ion channel would be greatly

Figure 2 Effect of hexamethonium on the relation between amplitude of endplate currents and membrane potential. Currents were recorded from one endplate in the absence (■) and presence (●) of hexamethonium 1 mM, applied in the solution bathing the muscle for at least 3 min.

reduced, and so any reduction of the ACh-induced conductance change may be attributed primarily to voltage independent antagonism.

Time course of endplate currents

Figure 1 also shows that while the amplitude of endplate currents was greatly reduced by hexamethonium, there was little change in their time course under these conditions (21°C, 1 mM hexamethonium). Semilogarithmic plots of the amplitude of endplate currents from Figure 1 a3 and b3 against time during their decay are illustrated in Figure 6a. There was no sign of a detectable fast phase of decay of the endplate current in the presence of 1 mM hexamethonium, although a small prolongation of the decay was observed at this membrane potential (−120 mV). The observation that hexa-

Figure 3 Effect of membrane potential on carbachol (CCh)-induced current in the presence and absence of hexamethonium (C6). The membrane potential was made to change from −60 mV to −120 mV and back to −60 mV at a steady rate by applying 'ramp' commands to the voltage-clamp, and is shown in the upper two panels. The current induced by 5 μM carbachol is shown in the lower left panel, and was obtained by subtraction of the current in the absence of carbachol from that in its presence (see Methods). The lower right panel shows that current induced by carbachol (80 μM) in the presence of hexamethonium (1 mM). Note that in the absence of hexamethonium the carbachol-induced current becomes more inward (downward deflection) with hyperpolarization, whereas in the presence of hexamethonium it becomes more outward with hyperpolarization.

Figure 4 Log(concentration)-response curves determined from carbachol-induced currents of the kind shown in Figure 3. Conductance was calculated from the carbachol-induced current assuming a reversal potential of 0 mV. The curves were determined at three membrane potentials: −60 mV (▲), −90 mV (■) and −120 mV (●). Note that hexamethonium, 1 mM, shifted the curves to higher carbachol concentrations, and that, in particular at −120 mV, the curve in the presence of hexamethonium was less steep than in its absence.

methonium did not increase the rate of decay of endplate currents under these conditions contrasts with observations of the effects of other drugs which show a voltage-dependent reduction in the amplitude of endplate currents (e.g. procaine, Katz & Miledi, 1975). It also differs from the findings of Milne & Byrne (1981) who observed biphasic decays of endplate currents in high magnesium (7.5–10 mM) Ringer solution at 13°C, and found that hexamethonium increased the rate constant of the early phase under these conditions. Since the observations shown in Figure 3 were made at room temperature (23°C), we repeated the experiment at 13°C to determine whether the difference in temperature could account for the discrepancy between our observations and those of Milne & Byrne (1981). There was no detectable increase in the initial rate of decay of endplate currents in the presence of hexamethonium (either 100 μM or 1 mM), and 1 mM hexamethonium caused a small

Figure 5 Effect of hexamethonium (C6) on equilibrium responses to acetylcholine (ACh) 10 μM. (a) Agonist-induced currents in response to bath application of ACh 10 μM at +50 mV (upper trace) and −50 mV (lower trace). Reversal (zero-current) potential, +3.1 mV. (b) Responses to bath application of ACh 10 μM and hexamethonium 200 μM at +50 mV (upper trace) and −50 mV (lower trace). Inward current plotted downwards. Note the calibration scale in (b).

prolongation similar to that observed at room temperature. The prolonging effect of hexamethonium on endplate current decay observed either at room temperature or at 13°C was dependent on the membrane potential. This is illustrated in Figure 6b where the time constant for decay of endplate currents recorded at 23°C is plotted semi-logarithmically against membrane potential for currents in the presence (squares) or absence (circles) of 1 mM hexamethonium. It can be seen that the prolonging effect of hexamethonium increased as the membrane potential was made more negative. A similar voltage-dependent prolongation of the decay of endplate currents by hexamethonium was observed consistently in all fibres studied over the membrane potential range of +60 mV to -140 mV. No component with an increased rate of decay was detected over this range of membrane potentials under the conditions of our experiments.

Fluctuation analysis of endplate currents

The mechanism underlying the prolongation of endplate current decay by hexamethonium was further investigated by measurement of agonist-induced current fluctuations (Anderson & Stevens, 1973) to distinguish between an effect of hexamethonium on acetylcholinesterase activity and/or the mean channel open-time ('burst length'; Colquhoun & Sakmann, 1983). Current fluctuation measurements were

made on the endplate voltage-clamped with the vaseline-gap method. The spectrum of current noise induced by acetylcholine alone is shown in Figure 7a; the noise was recorded during the plateau of response (an inward current of -30 nA) produced by bath application of 1 μ M ACh at a membrane potential of -100 mV. The theoretical Lorentzian curve fitted to the observed points related the single-sided spectral density, $G(f)$, to the frequency, f , by the relation

$$G(f) = \frac{G(0)}{1 + (f/f_c)^2}$$

which is defined by the parameters $G(0)$, the spectral density at zero frequency and the corner frequency, f_c , which is the frequency at which the spectral density is one half of $G(0)$. The noise spectrum (Figure 7a) is fitted by a single Lorentzian curve with a corner frequency, f_c , of 48.2 Hz which corresponds to a time constant, τ , of 3.3 ms for the mean channel open-time.

The presence of hexamethonium (500 μ M) produced a distortion of the spectrum of high frequencies (Figure 7b). The spectrum obtained at a membrane potential of -100 mV was fitted by a single Lorentzian curve ($f_c = 400$ Hz) giving a time constant, τ , of 4.0 ms; this corresponds to a 28% increase in the mean channel open time in the presence of 500 μ M hexamethonium. There was, however, a deviation of noise spectra from a single Lorentzian curve in the presence of hexamethonium, suggesting a second kinetic component of current

Figure 6 (a) Decay of endplate currents plotted semilogarithmically as a function of time; (●) in the absence of drugs; (■) in the presence of hexamethonium 1 mM, applied in the solution bathing the muscle for more than 3 min. (b) Voltage-dependence of the time course of endplate currents in the absence (●) and presence (■) of hexamethonium, 1 mM. Semilogarithmic plot of the time constant of decay of endplate current amplitude as a function of membrane potential. Temperature 20°C.

Figure 7 Power spectral density of endplate current fluctuations measured at -100 mV in the presence of acetylcholine (ACh) 1 μ M (a) and ACh 10 μ M plus hexamethonium 500 μ M (b). Each spectrum was fitted up to 1 kHz by a single Lorentzian curve (solid line) with the time constants calculated from the corner frequency indicated.

Table 1 Voltage dependence of the endplate channel 'apparent' conductance (γ) and mean open time (τ) in the presence of ($10\ \mu\text{M}$ ACh and $500\ \mu\text{M}$ hexamethonium) and absence of ($1\ \mu\text{M}$ ACh) hexamethonium

Membrane potential (mV)	γ (pS)	ACh $1\ \mu\text{M}$ τ (ms)	ACh $10\ \mu\text{M}$ + C6 γ (pS)	$500\ \mu\text{M}$ τ (ms)	$\tau_{\text{drug}}/\tau_{\text{control}}$
-70	26 ± 1	2.3 ± 0.16 (6)	19 ± 1	2.6 ± 0.3 (6)	1.13
-100	22 ± 1	3.6 ± 0.2 (5)	10 ± 1	4.8 ± 0.3 (4)	1.33
-130	25 ± 2	6.0 ± 0.3 (5)	6 ± 1	9.6 ± 1.4 (4)	1.60

Results of current fluctuation measurements made at 10 – 12°C given as mean $\pm$ s.e.mean. (n = number of experiments).

Ratios of mean channel open time (τ) in the presence and absence of hexamethonium are given in the right hand column.

fluctuations which is not adequately resolved in the present experiments. The effect of a fixed concentration of hexamethonium on the apparent mean open time became more pronounced as the membrane was hyperpolarized (Table 1). There was also an apparent reduction in single channel conductance in the presence of hexamethonium (Table 1), which may be attributed to an underestimation of the variance by failing to account for a second spectral component (see Figure 7b).

Effect of hexamethonium on agonist-induced currents in voltage-jump experiments

Microelectrode experiments The effects of membrane potential on agonist-induced currents (determined during the

plateau phase of response) were examined by applying step hyperpolarizations from $-70\ \text{mV}$ to between -100 and $-160\ \text{mV}$. Net agonist-induced currents obtained after subtraction of the control currents from those obtained in the presence of agonist are illustrated in Figure 8 for responses to $5\ \mu\text{M}$ acetylcholine alone (a) or for $15\ \mu\text{M}$ acetylcholine in the presence of $1\ \text{mM}$ hexamethonium (b). Neostigmine $3\ \mu\text{M}$ was present throughout to inhibit acetylcholinesterase. In the absence of hexamethonium, the acetylcholine-induced current at the beginning of the step increased with hyperpolarization as if there was an approximately ohmic increase of current through a fixed number of open ion channels as the driving force on ion flow was increased. This instantaneous current was followed by an exponential relaxation of the current to a new steady level, which corresponds to the new equilibrium number of open ion channels at the hyperpolarized membrane

Figure 8 Acetylcholine (ACh)-induced currents recorded from a single endplate immediately following a series of step changes in membrane potential. At the arrow indicating 'pulse on' the membrane potential was hyperpolarized from $-70\ \text{mV}$ to the potential shown with the current; at the arrow indicating 'pulse off' the membrane potential was returned to $-70\ \text{mV}$. The top panels (a) show currents induced by acetylcholine, $5\ \mu\text{M}$. The bottom panels show currents induced by acetylcholine $15\ \mu\text{M}$ in the presence of hexamethonium, $1\ \text{mM}$. Zero current is shown by the horizontal line at the top of each panel. Time constants for the slow component of relaxation were: $3.8\ \text{ms}$ ($-100\ \text{mV}$), $6.4\ \text{ms}$ ($-130\ \text{mV}$), and $7.9\ \text{ms}$ ($-160\ \text{mV}$) in the absence of hexamethonium; $4.4\ \text{ms}$ ($-100\ \text{mV}$), $6.6\ \text{ms}$ ($-130\ \text{mV}$) and $7.3\ \text{ms}$ ($-160\ \text{mV}$) in the presence of hexamethonium.

Figure 9 Relaxations of net agonist-induced current following step changes in membrane potential. Current relaxations for a 100 mV jump from and back to -50 mV holding potential during bath application of acetylcholine (ACh) $10 \mu\text{M}$ (a) and ACh $10 \mu\text{M}$ plus hexamethonium, $500 \mu\text{M}$ (b). Insert in (b): fast inverse relaxation following ohmic jump in current observed with voltage-jump to -150 mV in the presence of hexamethonium (τ_f , $90 \mu\text{s}$). Relaxations for voltage-jumps from $+50$ mV to -50 mV and back in the presence of ACh $10 \mu\text{M}$ (c) and ACh $10 \mu\text{M}$ plus hexamethonium (C6) $200 \mu\text{M}$ (d). Insert in (d): small amplitude, inverse relaxation recorded in response to a hyperpolarizing jump to -50 mV in the presence of hexamethonium $200 \mu\text{M}$ (τ_f , $200 \mu\text{s}$). The arrowheads indicate the (ohmic) instantaneous current.

potential. In the presence of hexamethonium the acetylcholine-induced current did not show an ohmic increase at the beginning of the step hyperpolarization, but rather decreased as the voltage was stepped to more negative potentials. The current then relaxed to a new equilibrium level, but this was reduced with more negative steps, in contrast to that observed in the absence of hexamethonium. For the example in Figure 8, the time constant for the slow relaxation in the

Figure 10 (a) Slow relaxation time constant(s) as a function of hexamethonium concentration at three different membrane potentials. (b) plot of $(\tau_f^{-1} + \tau_s^{-1} - \tau_c^{-1})$ against hexamethonium concentration at -150 mV. Pooled data obtained from voltage-jump experiments. Straight line is a regression fit to the data.

presence of hexamethonium was increased from a control value of 3.8 to 4.4 ms at -100 mV, whereas little or no prolongation was observed with steps to more hyperpolarized levels. It should be borne in mind that 'buffered diffusion' of acetylcholine in the synaptic cleft may complicate the estimation of such rate constants under the conditions of these experiments (see for example, Armstrong & Lester, 1979).

Figure 8 also shows the response evoked when the membrane potential was stepped back from -160 mV to -70 mV. In the presence of ACh alone, there was an initial ohmic step that was followed by an exponential decay of current back to the original holding level. However, in the presence of ACh plus hexamethonium, the current immediately after the depolarizing step was greater than the equilibrium level seen at the hyperpolarized level. The current then decayed to the new steady level.

Vaseline gap experiments It seemed possible that the non-ohmic behaviour of the ACh-induced current at the beginning of the voltage jumps in the presence of hexamethonium might reflect a voltage-dependent antagonism (block) which was too rapid to resolve with the two-electrode voltage clamp. Similarly the initial large current seen immediately after the hyperpolarizing pulse could reflect a rapid unblocking of membrane channels. This hypothesis was tested using the vaseline-gap voltage clamp method which allows resolution of current transitions in the microsecond range. These experiments were done on the extrajunctional region of denervated muscle fibres to avoid concentration transients which could arise within the synaptic cleft during a hyperpolarizing jump (Armstrong & Lester, 1979; Colquhoun & Sheridan, 1982). The use of denervated muscle also avoids the need to inactivate the enzyme acetylcholinesterase, which is absent in extrajunctional regions of frog muscle (Katz & Miledi, 1964; Miledi *et al.*, 1984).

Figure 11 Inward currents in response to acetylcholine (ACh) $10\ \mu\text{M}$ (a) and ACh $100\ \mu\text{M}$ plus hexamethonium (C6) $1\ \text{mM}$ (b) at $-50\ \text{mV}$. The duration of bath application of the agonist is indicated by the line above the current records. The time lag between application of the agonist and the start of the response is due to the 'dead-space' time between the solution inlet valve and for the solution change to reach the muscle membrane. Superimposed downward deflection on current records are responses (attenuated by pen recorder) to hyperpolarizing voltage-jumps to $-100\ \text{mV}$ and $150\ \text{mV}$. In the upper trace of (a) the position of the control voltage jumps, imposed in the absence of agonist, are also shown. Semilogarithmic plot of the decay of the current in (a) and (b) are shown below the corresponding record. Time constants of each exponential component of the current decay (desensitization) observed in (a) and (b) are given.

The relaxations of the net current induced by $10\ \mu\text{M}$ ACh under these conditions (Figure 9a) were fitted by a single exponential curve with a time constant of $2.7\ \text{ms}$ at $150\ \text{mV}$ and $2.3\ \text{ms}$ at $-50\ \text{mV}$. In this, and other experiments of this series, the acetylcholine concentration was kept constant in the presence and absence of hexamethonium. In the presence of hexamethonium ($500\ \mu\text{M}$) the shape of the relaxations is changed (Figure 9b). Hyperpolarization caused the expected ohmic jump (which had not been detected with the two-microelectrode clamp described above) followed by an initial

rapid decrease in inward current and then an increase which was slower than that in the control. The current during the first millisecond following each voltage step was sampled at a higher rate ($64\ \text{kHz}$) and is shown on an expanded time scale in the insert of Figure 9b to show clearly the rapid, inverse relaxation following the ohmic jump in current at $-150\ \text{mV}$. The relaxation following the voltage jump to $-150\ \text{mV}$ in Figure 9b was well fitted by two exponentials with time constants, $\tau_f = 90\ \mu\text{s}$ and $\tau_s = 32.8\ \text{ms}$.

When the membrane potential was stepped back from -150 to $-50\ \text{mV}$, the current seen in the presence of hexamethonium did not show the exponential decrease seen with the agonist alone (Figure 9a) but rather a rapid increase in current followed by an exponential decay in the normal direction. The rate of decay was again slower ($\tau_s = 11.7\ \text{ms}$) than in the control ($\tau_s = 2.3\ \text{ms}$). The amplitude and rate constant of the fast relaxation were dependent on the hexamethonium concentration and increased with higher concentrations of hexamethonium. When the concentration of hexamethonium was $> 500\ \mu\text{M}$, the time course of the relaxation following the $100\ \text{mV}$ depolarizing step was invariably too rapid to be resolved, even when sampled at $64\ \text{kHz}$.

Similar observations were also made when hyperpolarizing voltage-jumps were applied from a holding potential of $+50\ \text{mV}$ (Figure 9c and d).

The effect of hexamethonium concentration on the slow relaxation time constant at membrane potentials $-50\ \text{mV}$, $-100\ \text{mV}$ and $-150\ \text{mV}$ is shown in Figure 10a. Acetylcholine concentration was kept constant in the presence and absence of hexamethonium. As the hexamethonium concentration was increased above $200\ \mu\text{M}$ the slow relaxation time constant measured at all voltages was independent of antagonist concentration. A similar observation has been made for the concentration-dependence of the slow relaxation time constant recorded in the presence of decamethonium (Adams & Sakmann, 1978; Adams & Colquhoun, unpublished). Over the voltage range illustrated, an e-fold increase of the rate constant of the slow relaxation (τ_s^{-1}) required an approximately $+67\ \text{mV}$ change in membrane potential.

The sequential model for open channel block (Adams, 1976) predicts that, in the presence of a drug, the agonist-induced current relaxes as the sum of fast and slow exponential components whose rates are, respectively, increased and decreased linearly with increasing drug concentration. As with the observations on endplate currents reported above, this prediction was not fulfilled for the relationship between τ_s and hexamethonium concentration (Figure 10a). A second prediction of the model is that the sum of the fast and slow rate constants (reciprocal time constants) minus the control rate constant should depend linearly on hexamethonium concentration according to:

$$\tau_f^{-1} + \tau_s^{-1} - \tau_c^{-1} = [\text{H}]k_{+1} + k_{-1},$$

where τ_f = time constant for the fast component of decay, τ_s = time constant for the slow component of decay in the presence of hexamethonium, τ_c = time constant of decay in the absence of blocking agent, $[\text{H}]$ = concentration of hexamethonium, and k_{+1} and k_{-1} are the forward and backward rate constants for the blocking reaction. A plot of $(\tau_f^{-1} + \tau_s^{-1} - \tau_c^{-1})$ against hexamethonium concentration is shown in Figure 10b for results obtained at a membrane potential of $-150\ \text{mV}$. The data, though dominated by the value obtained for τ_f^{-1} , were fitted by a linear regression line; the slope of the line gives an estimate of k_{+1} of $1.2 \times 10^6\ \text{s}^{-1}$, and the intercept yields a value, k_{-1} , of $3400\ \text{s}^{-1}$. The apparent equilibrium constant, K , for the binding of hexamethonium to its channel blocking site is given by: $K = k_{-1}/k_{+1} = 283\ \mu\text{M}$ at $-150\ \text{mV}$. (This estimate should be regarded with some caution since a more complex model in which blocked channels may close would not be expected to give a linear relationship for Figure 10b). The rate constants of the two kinetic components (and consequently the equilibrium constant also) showed a strong dependence on

membrane potential (see Figure 10a). However, our inability to resolve the fast, inverse relaxations during voltage-jumps to potential less negative than -150 mV for all hexamethonium concentrations tested precluded any quantitative analysis of the voltage-dependence of the association (k_{+1}) and dissociation (k_{-1}) rate constants and hence equilibrium constants (K). If we assume similar voltage-dependent rates of association and dissociation to those measured for open channel block by gallamine (Colquhoun & Sheridan, 1981) or QX222 (Neher & Steinbach, 1978), the equilibrium constant for the binding of hexamethonium to the ACh-activated channel at membrane potential of -70 mV would be expected to be approximately 2 to 3 mM. These values for the equilibrium constant are slightly greater than the concentrations required for 50% block of endplate currents at -70 mV (see Figure 2).

Desensitization in the presence of hexamethonium

Another feature of ACh responses in the presence of hexamethonium was noticed during the course of these experiments. In the records shown in Figure 11 the peak amplitude of the response (-110 nA) to $10 \mu\text{M}$ ACh was mimicked by increasing the ACh concentration to $100 \mu\text{M}$ in the presence of 1 mM hexamethonium. These comparable currents may reflect similar receptor occupancy by ACh, and comparison can be made between the rates of desensitization of the response in the presence and absence of hexamethonium. The decay of these inward current from their peaks was biphasic and best fit by the sum of two exponentials. The similarity between these rates of onset of desensitization in the presence and absence of hexamethonium at comparable peak inward currents is consistent with the suggestion that hexamethonium does not significantly enhance or diminish the degree of desensitization (cf. Rang & Ritter, 1970; Terrar 1974).

Discussion

The main findings of the present study are that hexamethonium reduces the amplitude of endplate currents in a voltage-dependent manner while only minor changes are detectable in the time course of endplate currents. The observations from 'voltage-jump' studies are compatible with a rapid and voltage-dependent blocking action by hexamethonium of ACh-activated channels, and the question arises whether this action would be sufficient to account for the observed effects on endplate currents.

In mammalian ganglion cells, there is good evidence that hexamethonium blocks ion channels associated with nicotinic receptors (Ascher *et al.*, 1979; Gurney & Rang, 1984; see also Blackman, 1959 and Blackman & Purves, 1968). The action of hexamethonium in skeletal muscle is less clear. Milne & Byrne (1981) concluded that part of the action of hexamethonium on frog skeletal muscle was similar to some other drugs that are thought to block ion channels, for example procaine (Adams, 1977), in that the decay of endplate currents in the presence of hexamethonium showed two exponential components, the faster of which is thought to reflect the forward reaction of binding to the open channel. However, with up to 1 mM hexamethonium under the conditions of our experiments, we have not detected a component of decay of endplate currents which is faster than that in the absence of drugs. Raising the external magnesium ion concentration has been shown to prolong e.p.c. decay as a consequence of an increase in the mean channel open time at the amphibian motor endplate (Takeda *et al.*, 1981; see also McLarnon & Quastel, 1983). This effect of high magnesium on end-plate channel kinetics may contribute to the appearance of a biphasic e.p.c. decay observed in the presence of hexamethonium by Milne & Byrne (1981).

The most direct evidence from our experiments for a channel blocking action of hexamethonium comes from the

voltage-jump experiments, where the rapid reduction in agonist-induced current following a step hyperpolarization in the absence of hexamethonium can be interpreted as a block by hexamethonium of open ion channels. Channel-block models also predict the observed rapid surge of inward current at the end of a step hyperpolarization (cf. gallamine, Colquhoun & Sheridan, 1981), the interpretation being that this represents rapid unblocking of the channels as the membrane potential is restored to its initial level.

Additional evidence consistent with channel-block is provided by further analysis of voltage-jump experiments where different concentrations of hexamethonium were used: it was shown in Figure 10 that the expression, $\tau_f^{-1} + \tau_s^{-1} - \tau_u^{-1}$, was approximately linearly related to the concentration of hexamethonium. According to the sequential channel block model (in which the blocking drug can only bind to and dissociate from open channels, see e.g. Adams, 1977), the slope of this relationship should be equal to the forward rate constant, k_{+1} , for the blocking reaction, and the intercept on the ordinate should be the backward rate constant, k_{-1} , for unblock. The estimates for hexamethonium were: $k_{+1} = 12 \mu\text{M}^{-1} \text{s}^{-1}$ and $k_{-1} = 3400 \text{s}^{-1}$. The equilibrium constant for the reaction between hexamethonium and ion channels, derived from the simple sequential model as the ratio of these two figures, is $280 \mu\text{M}$ at -150 mV.

There are, however, some discrepancies between our observation and the predictions of a simple sequential channel block model. If single channel behaviour is considered, the sequential model predicts that a blocking drug which dissociates rapidly from the channel (e.g. gallamine) would be expected to cause substantial prolongation of the time from first opening to final closure during a cluster of openings (see Colquhoun & Sheridan, 1981; and Ogden & Colquhoun, 1985). This in turn leads to a slow phase in decay of endplate currents, which was not pronounced in our experiments in which 1 mM hexamethonium caused less than a twofold prolongation even at -100 mV, and at -50 mV no prolongation was detectable. More complex models for channel block in which channel closure is not inhibited by occupation of the channel by the blocking drug have been described in detail by Colquhoun & Ogden (1985; see also Adams, 1977). One point arising from their discussion is that the mean open time per cluster is no longer independent of the concentration of blocking agent (since blocked-open channels may convert to blocked-shut channels, and then dissociation of the blocking drug may occur without re-opening). This in turn means that the prolongation discussed above need not be as great as would otherwise be the case. A further extension of this argument is that the total charge crossing the membrane during an endplate current appears to be reduced more in our experiments than would be expected on the basis of a sequential block. In addition, in the voltage-jump experiments the concentration dependence of the slow relaxation time constant is non-linear which is at variance with that predicted from the sequential model of channel block (see Adams & Sakmann, 1978).

If a more complex model for channel block (in which channels may close from a blocked state, see Ogden & Colquhoun, 1985) applies under the conditions of our experiments, the estimated equilibrium constant of $280 \mu\text{M}$ calculated above for the binding of hexamethonium to the channel would have to be revised.

An interesting point in this context is the route taken by hexamethonium when it dissociates from the channel; in the case of the open channel complex it seems reasonable that following dissociation the hexamethonium would normally escape to the exterior of the fibre, but it remains possible that the dissociation could come about by entry of hexamethonium into the fibre. Permeation of ion channels by hexamethonium is consistent with the observations of Dwyer *et al.* (1980) and of Creese & England (1970). In the case of the closed channel complex with hexamethonium, it seems possible that the hexamethonium may not easily escape to the

exterior and that dissociation might normally be accompanied by entry of hexamethonium into the fibre. Gurney & Rang (1984) have discussed a similar cyclic model for block of ganglionic neurones by methonium compounds, and concluded that for compounds with nine or more carbons between the charged nitrogens, the blocking drug may inhibit channel closure, whereas with smaller compounds (e.g. hexamethonium) the drug may become trapped in the closed channel unless the drug is so small that it can easily escape to the interior of the cell. It seems possible that in the case of hexamethonium at the neuromuscular junction the blocking drug is small enough, not to inhibit substantially channel closure, and yet has a low enough permeability to cause block, while still being able to dissociate slowly by entry into the fibre. If this were the case for hexamethonium, it would account for the reduction in amplitude of endplate currents without substantial prolongation. It also seems possible that the forward rate constant for channel block with 1 mM hexamethonium (approximately $10,000\text{ s}^{-1}$, see above) may lead to a development of block during the rising phase of an endplate current (approximately $500\text{ }\mu\text{s}$), so that no fast phase of endplate current decay reflecting channel block is detectable. This is unlike the majority of blocking drugs e.g. procaine (Katz & Miledi, 1975) or gallamine (Colquhoun & Sheridan, 1981), and might be attributed, at least in part, to the low potency of hexamethonium: k_{+1} for hexamethonium is not substantially greater than k_{+1} for gallamine (Colquhoun & Sheridan, 1981), but the effective forward rate is much larger for hexamethonium because the concentration required for an action is so high (more than 100 times greater than the concentration of gallamine).

There may of course be an additional effect of hexamethonium on receptors associated with ion channels. An upper estimate of such a possible effect can be derived from experiments at a membrane potential of $+50\text{ mV}$. At this potential the influence of the electric field would be to reduce the entry of hexamethonium into ion channels; however, there would still be a concentration gradient tending to drive hexamethonium into the channel and so even at this potential any residual antagonism may include a component of channel block. At $+50\text{ mV}$, the concentration of hexamethonium required to reduce the agonist-induced current to approximately 50% was $200\text{ }\mu\text{M}$: this may be compared with the dissociation constant of hexamethonium binding to acetylcholine receptors measured from the rate of inhibition of α -bungarotoxin binding to receptors in denervated rat muscle, which is approximately $100\text{ }\mu\text{M}$ (Colquhoun & Rang, 1976). Brenner & Micheroli (1985) also concluded that the action of hexamethonium at the frog endplate was in part a competitive block of receptors, combined with an action at ion channels which was not a simple sequential block.

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Another question which deserves attention is to what extent there may be an anticholinesterase effect of hexamethonium under the conditions of our experiments. Such an effect of hexamethonium on mammalian skeletal muscle has been described by Rang & Rylett (1984). In our experiments on frog skeletal muscle, a small prolongation of the decay of endplate currents was seen at a membrane potential of -120 mV , but unlike the effects of anticholinesterases such as neostigmine (Magleby & Stevens, 1972), the prolongation became less at more positive potentials, and was not detectable at -50 mV . The small prolonging effect of hexamethonium on endplate currents which we observed, and its dependence on membrane potential, may be compared with observations in experiments where acetylcholine-induced current 'noise' was measured: hexamethonium caused a small shift in the power spectrum of the noise at -100 mV towards lower frequencies (the reduction in the corner frequency, like the prolongation of endplate current decay at this potential, was slightly less than a factor of two), but no such shift was observed at -50 mV . The increased concentration of acetylcholine in the presence of hexamethonium might lead to an abbreviation of channel lifetime (when ACh concentrations are greater than the 'low concentration limit', see e.g. Anderson & Stevens, 1973); if such an effect were to occur it would lead to underestimation of the apparent prolongation in the presence of hexamethonium. Inhibition of acetylcholinesterase would not be expected to modify the power spectrum for acetylcholine-induced current noise because the lifetime of occupied open channels would not be expected to be lengthened by the action of an anticholinesterase (which would be expected only to influence the time for which acetylcholine molecules are present in the synaptic cleft). It seems unlikely that an anticholinesterase effect of hexamethonium is a major cause of the small prolongation of endplate current decay and the shift in the power spectrum at more negative potentials. It may be that the small prolongation at negative potentials reflects an interference with the normal closure of ion channels, in the manner suggested above for the action of larger channel blocking drugs.

In summary, the results are consistent with a block by hexamethonium of ACh-activated ion channels, and it appears that this block may not prevent the presumed conformational change in the channel which in the absence of hexamethonium normally leads to channel closure. Thus, it appears that hexamethonium can cause a voltage-dependent reduction in the amplitude of endplate currents without a substantial effect on their time course of decay.

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